



Designing Polymeric Microfluidic Platforms for Biomedical Applications

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Designing Polymeric Microfluidic Platforms for Biomedical Applications

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A thesis submitted for the
degree of

Doctor of Philosophy

21.11.2011

Preface

This thesis is submitted as a partial fulfillment of the requirements for obtaining the PhD degree from the Technical University of Denmark. The research report has been conducted at the Department of Micro and Nanotechnology at DTU from the period of Jul 2008 to Nov 2011. The project has been supervised by Associate Professor Winnie Edith Svendsen, Professor Jennie Emnéus, Associate Professor Maria Dimaki and Arto Heiskanen, Post Doc. The project was funded by FTP and the European project, Excell.

This dissertation would not have been possible without several individuals who were always there extending their help in one way or the other to complete the work. First and foremost, I am grateful to my supervisor Dr. Winnie Edith Svendsen, who gave this wonderful opportunity to pursue my PhD to this level. I will never forget her concern and complete support through all the circumstances during the research.

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I also would like thank our EXCELL collaborators, Proffessor Merab Kokaia and Associate Proffessor Alberto Martínez-Serrano for their involvement and providing all the biological samples for the research.

My special thanks to my colleague Pranjul Shah, for his enthusiasm in sharing knowledge and assistance in lab work. Also, thanks to Dorota Kwasny for assisting me with FISH experiments. I wish she could have been in the project since its inception. A special thanks to Azeem for lending his laptop.

Thanks to my colleagues Luigi Sasso, Romen, Jacob Morresso, Patricia Vasquez, Casper Claussen, and Lars Andersen for their valuable scientific discussion and a friendly disposition. A special thanks to Natalia and Tania for helping me with tissue culturing.

I owe my deepest gratitude to my family for their constant encouragement. I thank my father who gave me an opportunity to discover myself and pursue my dreams staying out of all the family responsibilities.

I am pleased to thank NaBIS for the fantastic and friendly working environment that they provided. Thanks also for all their eggless cakes.

I am thankful to Jay and Susan for helping me with proof reading, formatting, and above all for their moral support. Last but not the least, I thank all my friends Abi, Lesitha, Sudhan, Vidhya, Sankar, Sujata, Niki, Basil, Uma, Honey, Viduthalai, and Lokanathan, who have always been in my life extending their support.

Abstract

Micro- and Nanotechnology have the potential to offer a smart solution for diagnostics and academia research with rapid, low cost, robust analysis systems to facilitate biological analyses. New, high throughput microfluidic platforms have the potential to surpass in performance the conventional analyses systems in use today. The overall goal of this PhD project is to address two different areas using microfluidics :

i) Chromosome analysis by metaphase FISH such a platform, if successful, can immediately substitute the routine, labor-intensive, glass slide-based FISH analyses in Clinical Cytogenetics laboratories. During the course of this project, initially the suitability of the polymeric chip substrate was tested and a microfluidic device was developed for performing interphase FISH analysis. With this device, the key factors involved in chromosome spreading crucial to FISH analysis were further investigated. Based on the insights gained, a micro splashing device was designed to achieve well-spread chromosomes and a rapidly assembled microFISH device was presented for metaphase analysis. Further, a single polymeric microfluidic device was developed to semi-automate the FISH analysis.

ii) Culturing brain slices and monitoring the integration of neuronal stem cells upon cultured brain slices. These studies will aid to design novel therapeutic approaches for neurodegenerative disease. The aim of this project was to create a microfluidic cell culture chamber and keep a brain slice alive in it for long time under stable conditions. Such a system was developed and tested first with PC12 cell line culture, followed by brain slice culture. This culture system was later adapted to suit long-term culturing and was successfully demonstrated.

Danish Summary

Mikro-og Nanoteknologi har potentiale til at være en smart løsning indenfor diagnostik og den akademiske forskning med hurtige, billige, robuste analyse-systemer til biologiske analyser. Ny, højkapacitets mikrofluidiske platforme har potentiale til at overgå de konventionelle analysesystemer i brug i dag.

Det overordnede mål med dette ph.d.-projekt er derfor at tage fat på to forskellige områder ved hjælp af mikrofluidiske systemer:

i) Kromosomanalyse ved metafase FISH. Udviklingen af sådan et system kan umiddelbart erstatte rutine, arbejdskraftintensive, objektglas baserede FISH analyser i kliniske cytogenetiske laboratorier. I løbet af dette projekt, egnetheden af en polymer chip substrat blev testet i første omgang, og en mikrofluidisk enhed var udviklet til at udføre interfase FISH analyse. Med denne enhed, de vigtigste faktorer som er involveret i kromosom spredning, afgørende for en vellykket FISH analyse, blev yderligere undersøgt. Baseret på de erfaringer, der opnås, en spøjte chip er designet til at opnå gode kromosomspredninger og et hurtigt samlet mikroFISH chip for metafase analyse blev udviklet. Desuden var en enkelt polymert mikrofluidisk enhed udviklet til semi-automatiseret FISH analyse.

ii) Dyrkningen af hjernesiver, og overvågning af integreringen af neurale stamceller på dyrket hjerne-skiver. Disse undersøgelser vil hjælpe til at designe nye terapeutiske tilgange til neurodegenerative sygdomme. Målet med dette system var at holde en hjerneskive i live i en mikrofluidisk baseret kultur kammer i lang tid under stabile forhold. Sådan et system var udviklet og testet først med den PC12 celle linje, efterfulgt af en hjerneskive kultur. Dette kultursystem blev senere tilpasset så en skive kunne dyrkes i længere tid med succes.

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Abbreviations

BAC	Bacterial Artificial Chromosome
CGH	Comparative Genomic Hybridization
COC	Cyclic Olefin Copolymer
COP	Cyclo Olefin Polymer
C-TAS	Chromosome Total Analysis System.
DAPI	4',6-diamidino-2-phenylindole
DNA	Deoxyribonucleic acid
EXCELL	Exploring Cellular Dynamics at nanoscale.
FEP	Fluorinated Ethylene Propylene
FISH	Fluorescence <i>In Situ</i> Hybridization
GFP	Green Fluorescent Protein
GTG	G-banding by Trypsin using Giemsa
HPLC	High-Performance Liquid Chromatography
I-FISH	Interphase FISH
KCl	Potassium Chloride
LDH	Lactate dehydrogenase
L-DOPA	L-3,4-dihydroxyphenylalanine
LIC	LAB-on-a-Chip
M-FISH	Multicolor-FISH
MTT	Metabolic Toxicology Test
NaOH	Sodium Hydroxide
PBS	Phosphate Buffered Saline
PC	Polycarbonate
PCR	Polymerase Chain Reaction
PD	Parkinson's Disease
PDMS	Polydimethylsiloxane
PET	Polyethylene terephthalate

PMMA	Poly(methyl methacrylate)
PRINS	Primed <i>in situ</i> Labelling
RT	Room Temperature
SKY	Spectral Karyotyping
SSC	Saline-Sodium Citrate
YAC	Yeast Artificial Chromosome

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1. General Overview

1.1. Perspective of research

Lower survival rate has been reported for chromosome related diseases like cancer[1], where treatment is not always successful and obtainable therapy has some side effects[2-4]. It is crucial to diagnose the related chromosome abnormalities to provide an early treatment for such diseases. Estimated defective child birth, due to chromosome anomalies is more than 6% worldwide [5]. However, diagnostic techniques are still quite expensive, and there is therefore a need for a cheaper, fast, and sensitive technique for early diagnosis and better treatment. Recent developments in technology have reached another level with new screening platforms and analytical systems; however most current solutions are suffering from some challenges such as liquid handling and fast detection schemes that need to be dealt with.

Likewise, neurodegenerative diseases are common worldwide, and their causes are largely unknown. Detecting the dysfunction at the cellular level and with a higher sensitivity would help to develop novel therapies for such diseases. The results obtained by many available conventional methods are the average characteristics of a million cells together. To this end, it is necessary to address trace amounts of metabolites at a single cell level which would provide new insights into the underlying molecular mechanisms of disease. Diagnostic Medicine is also currently facing many challenges due to the lack of advanced technology to address single cell dynamics.

Micro- and nano- technology could address these issues by mechanical and electrical probing techniques [6-8]. Microfluidic platforms offer the advantage of handling reagents in small volumes making it a cost-effective yet high throughput technology[9]. This technology will revolutionize Medical diagnostics with new effective yet high throughput technology.

1.2. Objective of research

This thesis focuses mainly on two different areas; diagnosis of chromosome abnormalities and research to develop new therapeutic approaches.

The first research area aims at the development of a microfluidic device for Cytogenetics. The applications considered are pre- and post natal diagnosis and chromosomal anomalies in hematological malignancies. A technique called ‘FISH’ is used to detect the chromosomes abnormalities. We take a step towards miniaturization of the technique with microfluidic, which gives a cost effective, automated device for chromosome analysis.

The second research area aims at the development of a microfluidic system for monitoring the integration of stem cells in a brain slice culture. The primary goal was to develop a brain slice culture system for long term culturing. The culture system will later be integrated with a 3D electrode array chip to understand the cellular mechanism during integration of stem cells in host tissue during a therapy. However the development and integration of the 3D electrode chip is not the focus of this thesis.

All the chapters are interconnected to each other; therefore, some amount of repetition is to be expected.

1.3. Microfluidics

Microfluidics involves handling fluids in smaller geometric structures that facilitates the flow in a controlled manner. Fluids behave differently in a

smaller geometry that is in micro and nano level, unlike the macro level. The basic working principle of a microfluidic system is based on low Reynolds number resulting in a laminar flow profile. The mass transfer can be achieved either by pressure driven flow or by electro-osmotic flow.

In our work, we have used mainly pressure driven flow, which is achieved by commercially available syringe pumps or an in house Lego pump system[10]. The fabrication materials for the microfluidic chips are poly dimethylsiloxane (PDMS) and Poly methylmethacrylate (PMMA), polycarbonate (PC), Cyclic Olefin copolymer (COC) and Cyclo Olefin polymer (COP). All these materials have their pros and cons. We have tested the suitable material based on our analysis criteria (more details in chapter 3).

1.4. Development of microfluidic platform

1.4.1. Designing a microfluidic system

Designing the microfluidic chip is one of the important steps in developing a microfluidic device. Generally, a chip has a minimum of two layers, but several can be combined dependent on the application. The prototype can be

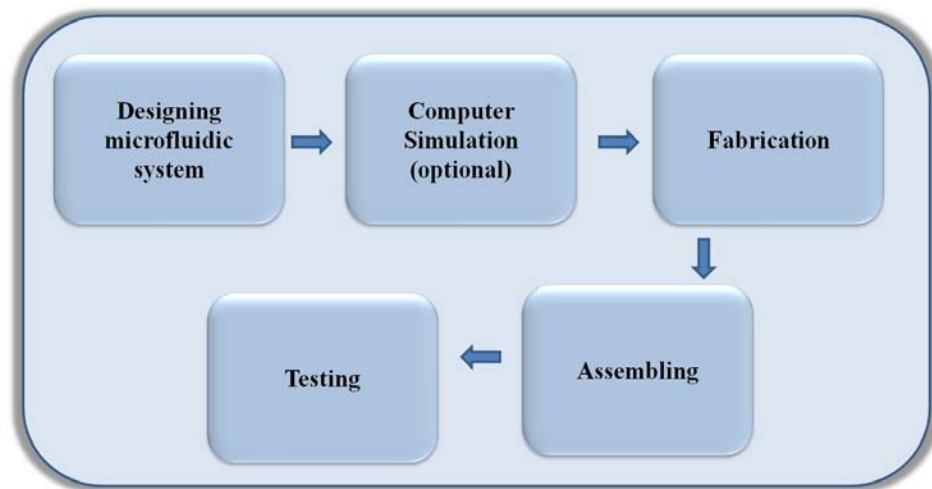


Figure 1.1: Basic principle of developing a microfluidic device

a very simple design to test the concept and also to reduce the fabrication time. However, if it involves complex designing, physical aspects of chip and behavior pattern can be predicted using a computer program, eg. COMSOL. According to the results obtained from COMSOL simulations the structural geometry can be re-modified.

In the case of micromilling, which is going to be used extensively in this work, AutoCAD is the commonly used software for 2D drawing the design which will further be translated into readable CNC code by EZcam software (computer numerical control). The specification of each layer has to be defined very precisely; otherwise, it can result in misalignment.

Polymers are well-suited for micromachining because of their unique qualities applicable to produce miniaturized biomedical devices. Polymer micromachining is less expensive than the silicon based fabrication process. Various polymers are commercially available in different forms and shapes. It is important to evaluate the polymeric qualities for fabrication of microfluidic devices, such as biocompatibility, transparency, chemical resistance, etc.

There are three types of polymers: elastomers (PDMS), thermoplastics (PMMA, COC, and polycarbonate), and thermal setting plastics. In this

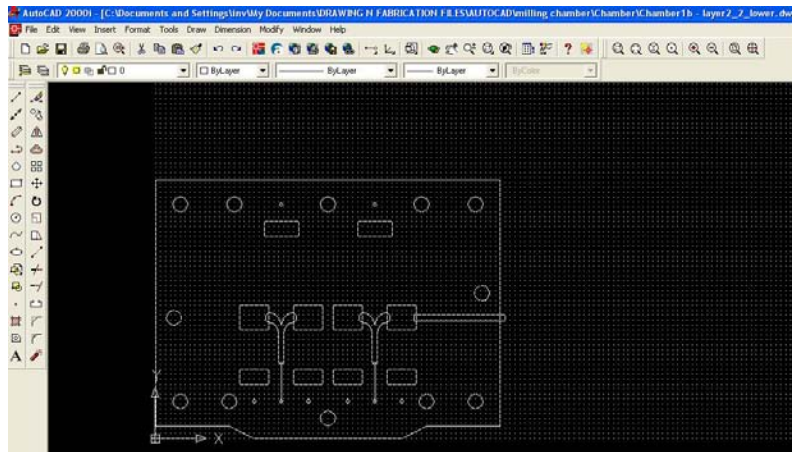


Figure 1.2: Designing a microfluidic chip by AutoCAD software.

thesis, elastomers and thermoplastics are mainly used based on their applications. Devices can easily be fabricated on these polymers by either laser milling or micromilling

Laser ablation

Laser ablation is an easy and fast method. It is very useful for making a prototype. It cuts the device using UV or infrared radiation. The UV beam by a CO₂ laser is focused on the polymer and the designed structure is written directly on the material. The UV radiation decomposes the polymeric materials due to thermal and chemical bond breaking. The polymer melts and leaves the residue near the cut edges. This method is not suitable for all thermoplastics such as COC and COP. It causes the formation of uneven surfaces, especially at the cut edges, which is difficult for bonding.

Micromilling

Micromilling is a mechanical method, in which the small cutting tools are used to remove the materials from a specific location. A computer is used to control the position of the tool (computer numerical control) and the cutting of the structures. Micromilling can be used only on hardened materials. The advantage of milling is that it will not affect the polymeric material by heating or UV radiation, but it creates stress near the cut structures that can be avoided by heating and slow cooling. The milling time can vary from minutes to hours dependent on the milling structure. The structure resolution depends on the tool dimension; it can go down to 25µm. However, using dimensions less than 200µm, carries the high risk of breaking the tool. All thermoplastics can be subjected to micromilling.

PDMS structure cannot be obtained directly by this method. The replica of the design can be machined and the mould can be casted in order to get PDMS devices. The moulds are reusable.

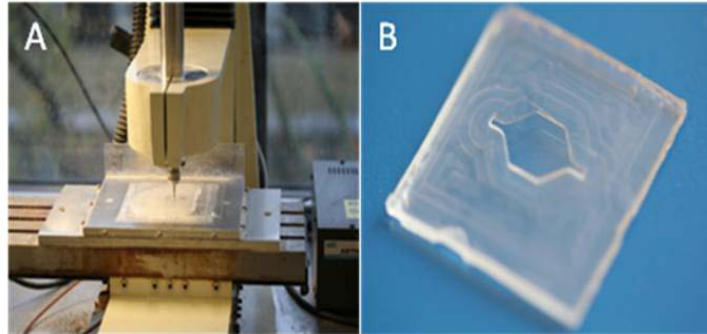


Figure 1.3: Image of milling fabrication instrument.
A) micromilling machine. B) A PDMS mould fabricated by micromilling.

1.4.2. Assembling

Fabricated chips have to be assembled to make a complete system. Usually a chip has a minimum of 2 layers, which are bonded together either by UV assisted thermal bonding or chemical bonding. The surface of the chip is activated by UV or chemicals, further it will be compressed at a particular temperature. The chip can either be integrated in a motherboard with fluidics connection or can be used as simple chip connected with tubing for fluidic supply.

1.4.3. Testing

The fabricated chip has to be tested in the lab to understand the accessibility for the applications. Common problems such as leaking and clogging may be encountered. This can be due to the bonding or structure misalignment. Other issues such as optical properties may be compromised during the assembling process. After testing, from the knowledge gained, sometimes the chip has to be redesigned to achieve better performance

1.5. Microfluidics in biomedical applications

Miniaturization has emerged promptly over the past decades. Many researchers then were skeptic about the practicality of microfluidics in real applications which is now changed due to latest developments in the field. PubMed search query indicates that the term ‘microfluidics’ is encountered in tremendous numbers of publications since 2001[11]. The number of patents related to microfluidics has increased from approximately from 25 (1998) to 350 (2004)[11]. Micro/nanotechnology comprises a multi disciplinary area including material science, surface science, chemistry, physics, and biology. The first microfluidic device was developed by Manz *et al.*, in 1980[12]. The device was high-pressure liquid chromatography (HPLC) using Si-Pyrex technology. It set a milestone for miniaturization, which led to numerous innovative advanced technologies.

Microfluidic chips were developed for a wide range of applications in the health sector; Medicine- cellular responsiveness of drugs [13-18], toxicology [19], novel drug delivery methods [20, 21], and therapeutic strategies for life threatening diseases such as cancer and diabetes, clinical diagnosis and lab experimentation processes such as Biochemical analysis [22-25], microfluidic PCR [26-28], whole blood sample preparation [29-32], DNA micro analysis [32], two dimensional electrophoresis [33], single cell analysis [34-36], bioreactors for adherent [37] and non adherent cells [38, 39].

A detailed review was published by Haeberle *et al.*, for available micro- and nanotechnology and its application[40].A few definitions for some specific devices are given below. Capillary action based devices: Liquids are transported within the micro-channel under capillary force [41]. These devices are useful in many biochemical analyses.

Integrated microfluidic system: It contains many microfluidic units such as fluidic channels, control channel, mixers, valves, pumps etc. The liquid

transportation occurs by pressure driven units or electroosmotic pump [42, 43].

Electro kinetic based systems: contain microfluidic platforms integrated with electrodes. The liquid transportation is based on applied voltage by external supply[44]. This method is exploited for partial manipulations and clinical diagnosis.

Droplet based systems: it is an interesting approach in the microfluidic world; a single droplet is used for biological analysis with the microfluidic structures. To avoid the disintegration of droplets, they are surrounded by air or oil. Droplet based systems are used in vast biomedicine and biotechnology applications[45, 46].

Having mentioned all the good aspects of miniaturization, most of the time academic and industrial research fails to reach commercialization or the end user. In the following a few examples of commercialized microfluidic systems

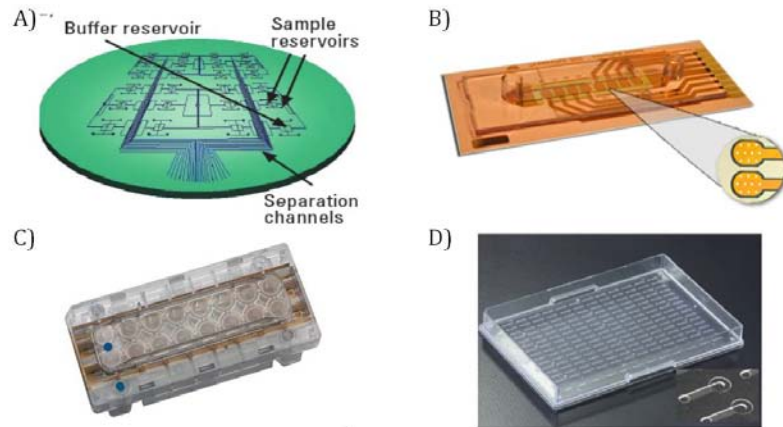


Figure 1.4: Microfluidic systems in biomedical applications.

A) 96 DNA sample -multiplex analysis system [47]. B) ECIS Electrode Arrays chip (Applied Biophysics Inc. USA, www.biophysics.com). C) CIM plate 16 is a single use device (ACEA Biosciences Inc. USA, www.Aceabio.com). D) The MC 5250 is multi channel array plate (Bell brooks lab, USA, www.bellbrooklabs.com).

for biomedical applications will be given.

A multiplex microsystem was developed for rapid PCR analysis products (Figure 1.4A) [47]. It has two sample reservoirs sharing the separation channel, a common buffer channel. PCR products are injected and separated by common channel as well.

The gold electrode array developed by Biophysics Inc. USA, shown in Figure 1.4B is a set of 8 circular electrodes with a diameter of 250 μ m. The array system is integrated with a polycarbonate substrate with insulating film. The channel area is 2.5 cm² and channel volume is 100 μ l. This system is used in impedance measurements. Endothelial cell behaviour is examined under flow conditions, similar to *in vivo* [48].

A system shown in Figure 1.4C consists of upper and bottom layers with 16 chambers, with a micro porous polyethylene terephthalate (PET) membrane in between. The bottom of the membrane includes microfabricated gold electrode array to monitor changes in cell migration. The lower chamber serves as reservoir for media and chemo attractant for the cells corresponding to upper chamber.

Multi channel array plate shown in Figure 1.4D consists of 192 micro channels, with the dimension of 1mm width x 0.14mm height x 5mm length. This device mimics the *in vivo* micro environment and was tested for cell/tissue culture to study stimulation and responsiveness in various cancer cells[49-51].

1.6. Thesis overview

These are discussed in details in the following chapters as follow. This chapter gave a little flavor of microfluidics and its potential applications in the biomedical field. The following chapters explain how we try to exploit microfluidics for FISH analysis and for monitoring the integration of stem

cells into brain slice. To present this work, the thesis is organized in two parts.

Part I (Chromosome Analysis by FISH Technique) consists of **Chapters 2, 3, 4, 5 and 6**. The work involved is organized in these chapters as described further. **Chapter 2** is an introductory chapter for this part of the research work. It provides an insight to the motivation behind the research presented in the subsequent chapters of this part of the thesis. **Chapter 3** describes experiments to characterize various polymeric materials with the intention of using them for fabricating microfluidic devices. These devices were used for interphase FISH analysis. As the chapters discussed, the aim of the analysis was to develop a chip for metaphase FISH analysis. However, since this was not as straightforward as it seemed, the research work progressed into understanding the factors influencing the dynamics of chromosome spreading in microfluidic devices. This phase of the study is presented in **Chapter 4**. With an understanding of the influential factors as identified in Chapter 4, the research led to the development of a microfluidic device for metaphase analysis. **Chapter 5** describes this device and the metaphase analysis activity. Finally, **Chapter 6** discusses and describes an approach to further optimize this device with the intention of semi-automation.

Part II (Monitoring neuronal stem cell/brain slice integration) consists of Chapters 7 and 8. This part of the research focused on studies to monitor the integration of neuronal stem cells into brain-slice. Such a study is especially useful in designing novel therapies for neurodegenerative diseases (e.g. Parkinson's disease). **Chapter 7** is an introduction to this part of the research. It describes existing techniques within the scope of this integration project. It describes especially neuronal stem cells; brain slice culture and review of existing brain slice culture chips. **Chapter 8** describes in detail the development process of brain tissue culture system and its testing. The experiments focus on culturing independent of CO₂ incubator and a detailed description of the experimental studies involving brain slices with syringe

pump and micropump. With this groundwork in place the chapter describes an experiment aimed at integrating neuronal stem cells and brain slices using a syringe pump.

Following the experiment studies and results presented in Part I and Part II of this thesis, **Chapter 9** presents a discussion and conclusion of the research results. It also provides an outlook of how the research can be taken further.

PART I: Chromosome Analysis by FISH Technique

2. Introduction to Chromosome Analysis by FISH Technique

2.1. C-TAS

The project “Chromosome Total Analysis System” (C-TAS) is aimed at detecting chromosomal aberrations using a miniaturized platform (Lab-on-a-Chip). It is a large project that has been divided into 3 different PhD projects.

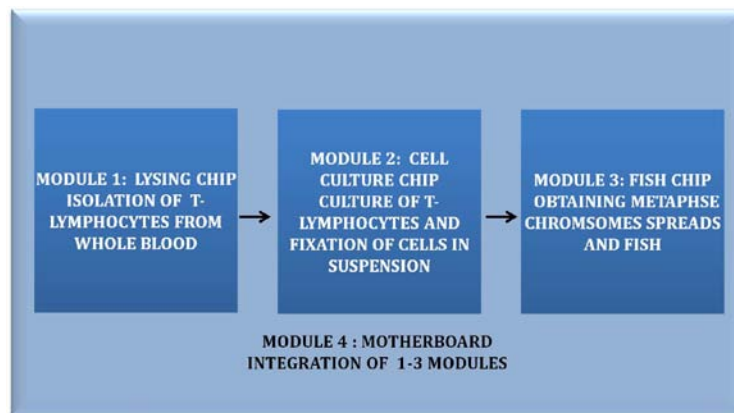


Figure 2.1 Finalized C-TAS modules for developing an integrated chip device.

The whole project is split up into 3 developmental modules which will be further integrated into an automated motherboard, as shown in Figure 2.1. In the first module, T-Lymphocytes (an easy source of chromosomes) are isolated from whole blood by lysing the red blood cells. In the second module,

isolated lymphocytes are cultured in a miniaturized bioreactor to get more metaphase cells. 3 days post culturing, isolated Lymphocytes will be arrested at metaphase and fixed in a fixative mixture. In the final module, the fixed metaphase cells are splashed and chromosome analysis will be performed. using traditional Cytogenetic tool such as FISH. But the ultimate aim of this part of the thesis is to develop a automated microfluidic FISH platform for clinical diagnosis of numerical and structural abnormalities.

2.2. Organization of Chromosomes

Cytogenetics refers to the study of chromosomes and Molecular cytogenetics refers to the study of genomic alterations using *in situ* methods. The first classical Cytogenetics technique ‘Karyotyping’ was developed in the 20th century, in which the chromosomes were stained in a unique pattern to detect the abnormalities in a whole set of chromosomes. In the 1980s, Fluorescence *in situ* Hybridization (FISH) was initially developed to detect chromosome rearrangements, mapping of human genes, microdeletions, and prenatal diagnosis of common aneuploidies in Clinical cytogenetics laboratories. Many FISH variants have continuously been developed including primed *in situ* labeling (PRINS), fiber FISH, Comparative Genomic Hybridization (CGH), Chromosome Micro-dissection, Spectral Karyotyping (SKY), Multi colour FISH (M-FISH), Color banding, FISH with multiple sub-telomeric probes, and array based CGH. All these techniques are mainly used in molecular cytogenetics. Now-a-days many commercialized FISH probes are available especially for applications in Cancer cytogenetics.

Deoxyribonucleic acid (DNA) is the heritable genetic material that influences the architecture and function of cells of human body. The DNA double helix is tightly coiled around histones, which are a group of proteins associated with DNA, and forms a superstructure known as ‘Chromatin’ (Figure 2.2) [52]. A chromosome is made up of this chromatin. Depending on the state of

the cell (undergoing cell division or not), the chromatin is either completely stretched out or curled up to some degree. During metaphase, the chromosome is highly condensed attaining the familiar x-shape with two genetically identical chromatids bound together at their centromere (the narrowest point on the chromosome).

The centromere consists of repetitive sequences, which increases the chances of a probe with the right sequence (a centromeric probe) to bind on to a specific region on the chromosome. There are 23 pairs of chromosomes located in the nuclei of all diploid cells, except for mature red blood cells which lack the cell nucleus.

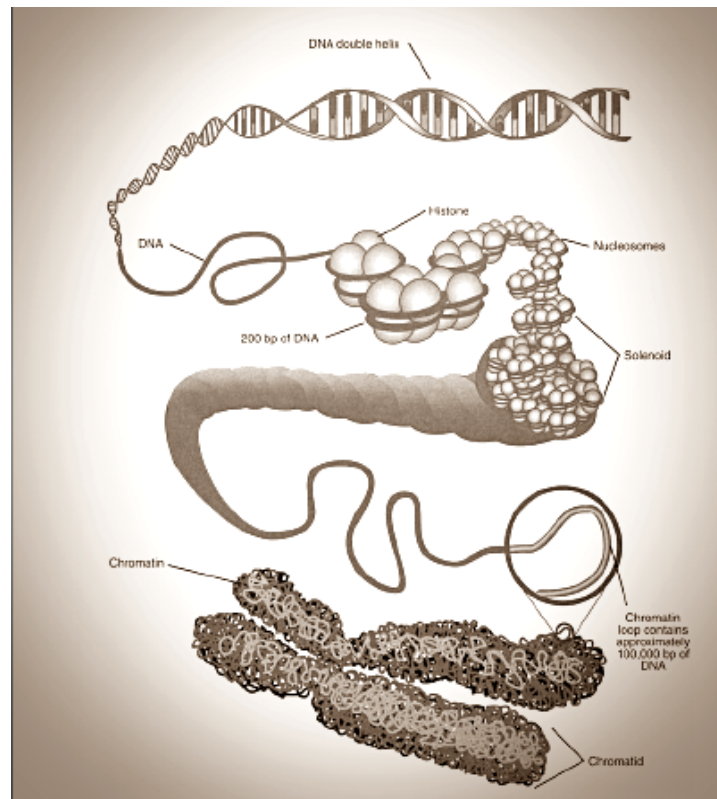


Figure 2.2: Organization of chromosomes in a cell nucleus.

2.3. Chromosome aberrations

Most of the eukaryotic cells undergo cell cycle, involves four stages; M, G₁, S, and G₂ (Mitosis, Gap 1, Synthesis (DNA replication) and Gap 2 respectively). During each mitosis process, a nuclear division occurs and two daughter cells

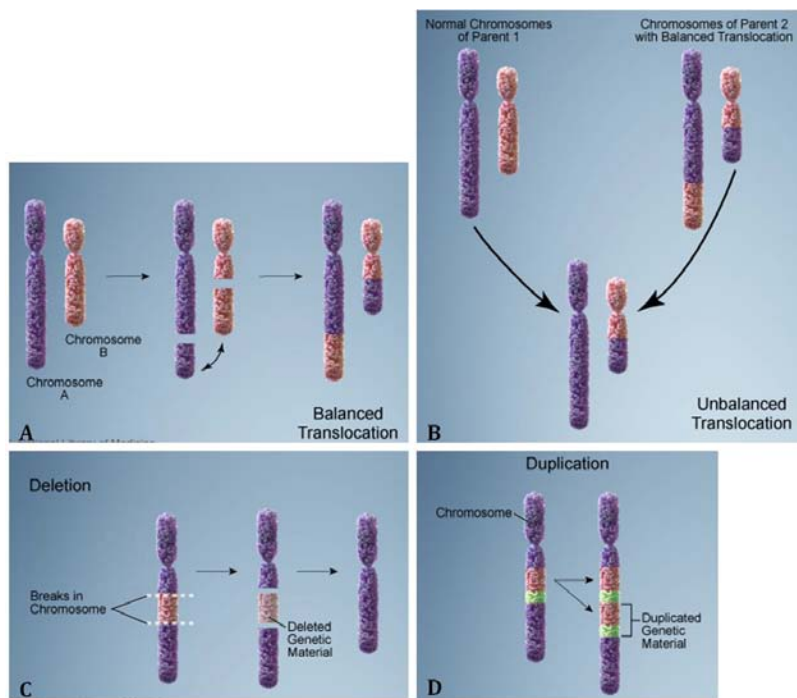


Figure 2.3: Types of chromosomal changes.

A) Balanced translocation, B) Unbalanced translocation. C) Deletion: Complete loss of portion of genetic material from a chromosome and D) Duplication: Gain/duplication of a part of genetic material. (www.ghr.nlm.nih.gov).

are formed. Mitosis is a dramatic event that divided into four stage; prophase, metaphase, anaphase, and telophase. In this basic process the chromosomes undergo fundamental changes such as condensation, spindle formation, attachment of chromosomes to the spindle fibres and finally movement

Introduction to Chromosome analyses FISH Technique

towards the poles and separation into two daughter cells. During synthesis the chromosomes are decondensed (interphase) and equally distributed in the nucleus. However, the mitosis process takes place for an hour and 95% of the cell cycle spends in the interphase stage. During the cell cycle, there are quite changes of mutation in the process, which leads to chromosome abnormalities.

Chromosomal translocations are defined as rearrangements between parts of chromosomes (Figure 2.3). In balanced translocation, exchange of genetical material does not affect the gene and its function. Whereas, in unbalanced translocation the gene is affected (more than 100 identifiable genetic syndromes such as leukemia, infertility, Down syndrome, Turner syndrome etc). The rate of chromosomal abnormalities in live births is estimated at

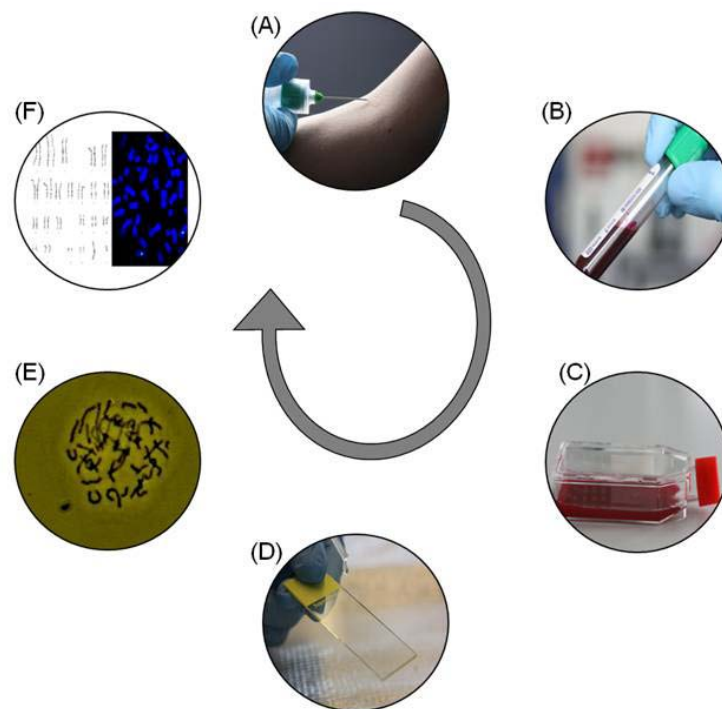


Figure 2.4: The cytogenetics analyses protocol.

Blood sample is collected from the patient (a) The collected blood (b) is cultured in a medium for 3 days at 37 °C (c) Colchicine is added at the end of the culture period to arrest the cells in metaphase. Followed by hypotonic treatment, the cells are splashed on a glass slide (d) to obtain metaphase chromosome spreads (e) At this point the chromosome spreads can either be analyzed by banding or FISH.

0.7 % [53]. To this end, cytogenetic analyses are extremely important for pre and postnatal diagnostics. Molecular genetics is one of the fastest growing biomedical fields for the diagnosis of genetic syndromes.

Cytogenetic analysis starts with obtaining the cells as a chromosome source. The most commonly used cells for cytogenetic analysis are peripheral T-lymphocytes, because they can be obtained from peripheral blood samples (Figure 2.4A). To perform analysis on metaphase chromosomes, the cell culture is required to increase the amount of cells in metaphase, which is not necessary for interphase analysis. Phytohaemagglutinin is typically used to stimulate cell division during a 72 hours T-lymphocyte culture (Figure 2.4B&C). A minimum of 100 cells need to be analyzed to obtain reliable results [54]. A mitogenic stimulation with Colcemid is further used to arrest the cells at metaphase and thus increase the number of metaphase plates for the analysis (Figure 2.4D). After treatment with hypotonic solution (Figure 2.4E), the cells are splashed on the glass slide with ice-cold water to obtain the metaphase plates (Figure 2.4F). Karyotyping by banding relies on staining these metaphase plates fixed on a slide surface and further analyzed under a microscope, while FISH requires the use of fluorescently labeled probes. FISH is based on hybridization between the DNA template and its complementary probe sequence, which is fluorescently labeled.

The fluorescent microscopic visualization of a hybrid between a probe and the template reveals the presence, number, and distinct location of target sequences [55, 56].

Over the past years, cytogenetic techniques have been evolving to enable more accurate and faster detection of chromosomal abnormalities. Nevertheless, the conventional Karyotyping by banding technique is still a standard procedure in cytogenetic laboratories over the world despite the need for trained technical staff [57-59]. Such a simple but time-consuming analysis allows for inexpensive diagnosis of numerical and structural chromosome aberrations at a limited resolution [60, 61]. The main advantage

of Karyotyping by banding is that it is a whole genome screening technique, where the entire genome can be visualized at once [62]. The requirement for high quality metaphase chromosome spreads for Karyotyping is challenging, as some cells (solid tumor cells) cannot produce good spreads [59].

FISH technique is used to overcome issues associated with such as limited resolution, requirement for cell population, and quality metaphase plates. The conventional banding analysis can only be carried out on metaphase chromosomes, while FISH may be performed both on metaphase and interphase chromosomes. Introduced over 30 years ago, the FISH technique commenced a new era in the cytogenetic studies providing greater resolution than was possible with the Karyotyping by banding technique [57, 63-65]. The typical resolution obtained with FISH is in the range of 10-100 kilo bases. While Karyotyping by banding has a resolution of 5-10 mega bases [57, 60, 65, 66]. FISH provides a simple, fast and reliable means to assess genetic instability in cancer [53, 67-69]. Despite many advantages of performing FISH, it is not an alternative to Karyotyping but a complementary technique.

2.4. Metaphase FISH

One of the attractive features of FISH is that it can be performed both on metaphase chromosomes and also on interphase nuclei fixed on a glass slide providing valuable information about chromosomal abnormalities [60, 68]. Interphase FISH has a great potential for screening large number of cells as well as for examining archival samples. Interphase FISH is used to identify numerical abnormalities as well as specific (known) structural abnormalities by targeting them with specific DNA probes. Metaphase chromosomes are widely used in cytogenetic analysis not only for standard Karyotyping but also for FISH analysis. FISH can also be used on metaphase cells to detect specific microdeletions at high resolution.

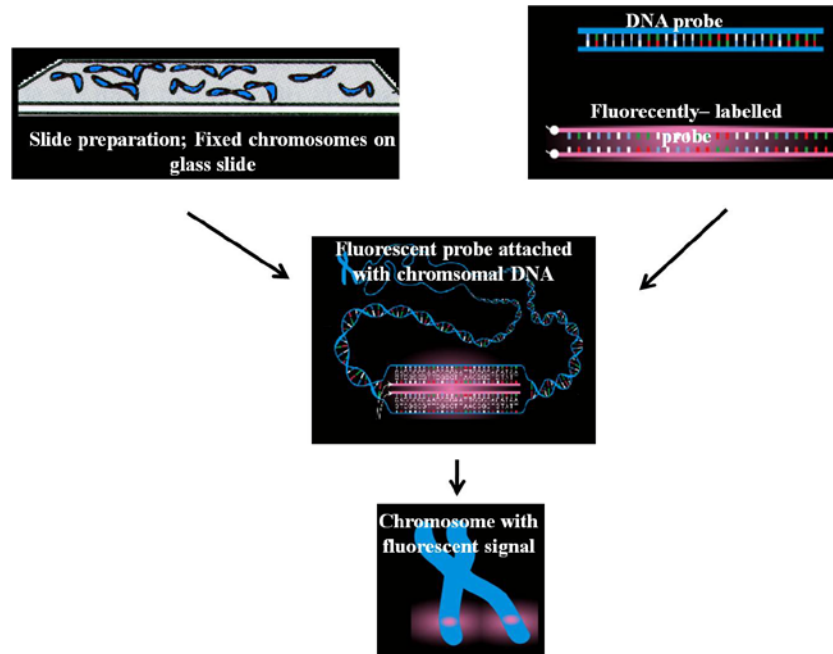


Figure 2.5: Metaphase FISH protocol.

Chromosomes are fixed on the slides using fixative; simultaneously the probe is designed for target sequence that is fluorescently labeled. The probes are applied on the slide and sealed tightly with cover slip using special glue. Temperature is increased to 75°C for 3-5 min to unwind the chromosomal DNA. Temperature is reduced and maintained at 37 °C overnight for hybridization. Next day, after few washing steps (to remove non-specific and loosely bound probes) the signals (pink dots) can be obtained by epifluorescence microscope. The chromosome is stained with counter stain, DAPI. This figure is modified from Jain *et al.*, 2007 [65].

It is also applicable in cases where it is troublesome to determine whether a chromosome has a simple deletion or is involved in a more complex rearrangement. Using metaphase chromosomes for analysis is beneficial as one can visualize the entire genome at once. The steps involved with metaphase FISH assay are shown in Figure 2.5. Whole chromosome FISH probes, a complex mixture of sequences from the entire length of each of the 23 pairs of

chromosomes that enables detecting abnormalities at the genome level [70]. Simultaneous visualization of spectrally distinct fluorophores enables the representation of different chromosomes with a unique fluorescent signature with a potential application to reveal complex structural abnormalities [58, 60-62, 68, 71, 72]. By performing such analysis, we can obtain information about the unknown translocations between chromosomes at higher resolution than achievable by karyotyping by banding [68, 73-75]. However, such method requires specialized computer software, expensive equipment, and complex analysis by skilled technicians that can interpret the obtained data [62].

The major disadvantages of these conventional techniques are that they are very expensive, labour intensive, and time consuming. These issues could be addressed by novel technology that could significantly increase the clinical utility of these techniques in diagnostics.

2.5. Microsystems developed for FISH analyses¹

In recent years the integration and automation of cytogenetic techniques has gained more attention. Most reports in this field focus on the development of an integrated microfluidic chip for interphase FISH analysis. To our knowledge there has been only one report on a microfluidic platform for metaphase FISH on chip. This article reviews the recent developments in the field of microcytogenetics that address the need for automation, time and cost reduction in chromosome analysis. In 2007, Lee *et al.*, presented one of the first examples of miniaturized devices for performing FISH analysis [76]. Their work allows for high throughput FISH analysis on a cell array. This analysis lacks the possibility of conducting high throughput FISH analysis on a cell array as it is not feasible in case of limited amount of samples for analysis. To address this problem, the authors showed the possibility to array cells by spotting small amounts of cell specimens onto a supporting matrix which enables high throughput analysis. For the preparation of the chip, a glass slide was used as a supporting matrix. 1 mm thick perforated PDMS was bonded to a glass slide to form 96 cavities of 1.5 mm diameter for spotting cells (Figure 2.6) enabling microscopic cell identification. A matrix² corresponding to the 96 PDMS wells was microfabricated by photolithography on a glass slide. They have showed that 1 μ l samples were spotted onto each PDMS well followed by air drying, removal of PDMS cover, and performed conventional FISH analysis, thus only 10 μ l of probe were used to analyse 96 specimens. The device is very useful in mass sample

¹ Some of the material has been adopted from our previous unpublished data. Kwasny D, Vedarethinam I, Shah PJ, Dimaki M, Silahtaroglu A, Tumer Z and Svendsen WE. Recent developments of microtechnology based methods for detection of chromosomal abnormalities

analysis for the same kind of samples. The authors have mentioned the probe volume reduction, but other major issues such as manual intervention and time consumption were not stated.

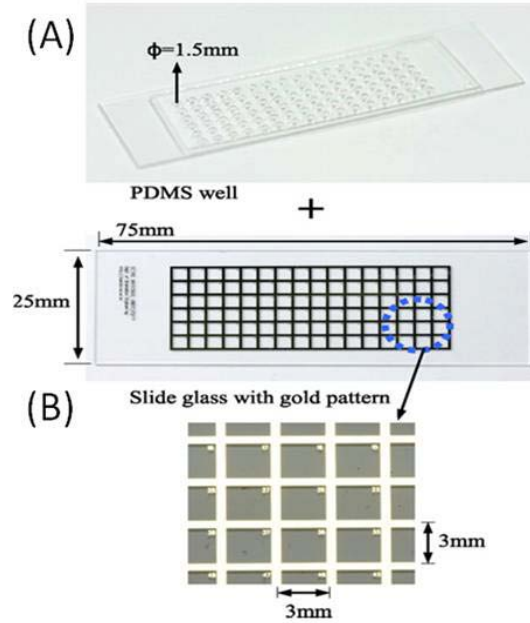


Figure 2.6 Fabricated bio-cell chip slide.

(A) A PDMS layer with 96 wells for multiple cell analysis.

(B) A glass slide with a gold pattern for sample numbering

Image is taken from Lee et al, 2007 [76].

The first implementation of FISH on chip was showed in 2007 by Sieben *et al.*, [77]. They have adapted the conventional FISH except the cell immobilization step, where cell immobilization was achieved using temperature. They also explored a method to reduce hybridization time by probe recirculation with on-chip peristaltic pumps, and electro-kinetic transport of probes by inserting electrodes into the end-wells. Of those approaches, it was found that electro-kinetic transport performed slightly better than recirculation. In addition, the electro-kinetic chip design is simpler

Introduction to Chromosome analyses FISH Technique

than the recirculating micro pump, which involves valves and complex micro fabrication. Nonetheless, by performing FISH on-chip at microfluidic volumes, they were able to achieve a tenfold reduction in DNA probe consumption per test, with corresponding cost reduction from \$90 for 10 μL of probe, to \$9 for 1 μL .

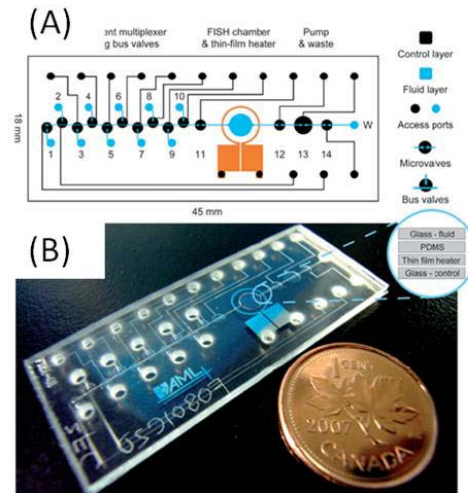


Figure 2.7: The mask layouts of an integrated FISH microchip.

The microchip includes a reagent multiplexer, a cell chamber with an integrated thin-film heater, and a peristaltic pump. (B) Image of a complete integrated FISH chip [77].

n 2008, Sieben, *et al.*, published another paper in which all previously performed steps with microfluidic FISH were integrated onto one chip and automated [42]. Similar to the re-circulating design, the new chip consisted of a PDMS layer sandwiched between two glass layers, one of which carried fluids, while the other transmitted air pressure for valve control, e.g. for peristaltic pumping, which is a very complex fabricated system. With the valves operated by computer, preloaded reagents could be multiplexed through the hybridization chamber at appropriate intervals for each step of FISH. A copper thin-film heater was built into the design for the cell-immobilization and denaturation (Figure 2.7). Unlike the previous designs, this chip did not include hybridization enhancements, but instead focused on minimizing technician labor time. Whereas conventional FISH takes more than an hour of on-and-off attention from a skilled technician, using their time inefficiently, automated microfluidic FISH reduced human intervention to only involve reagent preparation, and results analysis with fluorescence microscope.

The most recent advances in integrated interphase FISH analysis were presented in 2010 by Zanardi, *et al.*, [78]. They focused on the development of an analytical tool for performing interphase FISH on both living and fixed cells. The application of microfluidic technologies might reduce costs and improve assay performance by development of micro channels for easy reagent loading and cell immobilization. However, the fluid flowing in the micro channels causes intense shear stress on cells; this may result in their disruption or detachment from the surface. In this paper the authors' addressed this particular problem in the application of microfluidics for FISH.

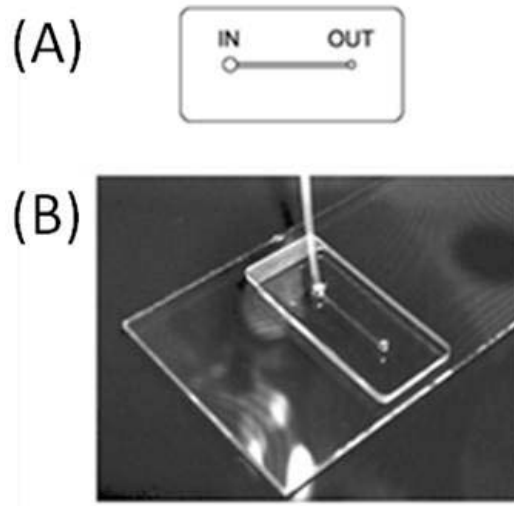


Figure 2.8: Microfluidic device for miniaturized FISH

A) Diagram of the microfluidic PDMS pad with inlet and outlet connected by a straight channel. (B) Complete structure of the FISH device with the PDMS pad assembled with a TiO_2 functionalized glass slide. Image is taken from Zanardi *et al.*, 2010 [78].

By changing the surface chemistry they enhanced the cell immobilization on the surface that significantly reduced the cells detachment due to shear stress. In this article, the entire glass slide is coated with nano structured TiO_2 using cluster beam technology which adds significant costs to the device owing to the need for access to cleanroom and costs of additional metal deposited. Moreover, routine genetic labs do not necessarily have access to cleanroom facilities, which makes the method very difficult to implement on a larger scale. The top cover bonded to the modified glass slide contains a microfluidic channel that is used for loading cells and other reagents in FISH protocol (Figure 2.8). Such a simple design facilitates handling of the fluids without generating bubbles that could affect assay performance.

The assays and devices presented in this review can be easily incorporated in cytogenetic laboratories to improve the quality of analysis and reduce the time necessary to obtain significant results. All of the mentioned work was done to improve the interphase FISH. These platforms offer great advantages over conventional methods in regards to reduction of probe volume per analysis used as well as the possibility to automate the protocol. Nevertheless, there is still a gap between developing the technology and usage of these systems as a routine diagnostic tool in cytogenetic laboratories. And also, there is a great need for rapid and inexpensive detection of unknown translocations using metaphase analysis. This was our motivation to develop a low cost, automated microfluidic platform for detecting chromosome abnormalities. In the following chapters, we present the development of the microfluidic chip for metaphase FISH assay.

3. Characterization of Polymeric Materials for Diagnostic FISH

3.1. Introduction

FISH is a sensitive diagnostic cytogenetic tool to help visualize chromosomes and chromosomal translocations [55, 67, 79] in the genomic DNA. Metaphase FISH helps visualize the genomic location of target DNA sequences with resolutions ranging between 2-3 Mb. Metaphase FISH can be performed on unknown samples by generating precise probes. Commercial probes are not available for all chromosomal translocations. The probes can be derived from plasmid, cosmids, bacterial artificial chromosome (BAC) and yeast artificial chromosome (YAC)[67]. The FISH process includes immobilization of cells, probe labeling, RNase treatment, denaturation of chromosomal and probe DNA, hybridization, post hybridization wash and image processing. Even though FISH is a powerful tool, it is very expensive and reasonably time consuming. The traditional method should ideally be replaced by fast and low-cost methods.

Recently, polymers have gained more attention in the micro- and nano- world [13, 24, 29, 39]. They have several advantages over conventional glass such as reduced material costs, availability of various techniques for chip fabrication and rapid prototyping, and good optical properties [80]. Apart from these advantages, plastics have certain drawbacks such as reduced

chemical resistance, poor surface chemistry, thermal resistance, and auto-fluorescence.

Few investigations have been done until recently about the optical properties of plastic materials. Aigers *et al.*, showed that PDMS exhibits low auto fluorescence and that it exhibits slightly higher auto fluorescence in the chip rather than as raw material[81]. Hawkins *et al.*, has also investigated various polymers and their auto fluorescence properties[82]. They confirmed that prolonged laser excitation (bleaching) of polymers leads to a reduction in the auto-fluorescence thus enhancing the optical property. Yun Chen *et al.*, extensively reviewed the optical, mechanical, and chemical properties and ease of fabrication and modification of PMMA in microfluidics and concluded that PMMA has excellent optical transparency comparable to that of borafloat glass [83].

In this study we characterized different polymeric materials on the basis of their optical property and cell fixation capability to identify the optimal substrate for developing miniaturized FISH assay to replace conventional glass slides. The efficiency of detecting hybridization signals in FISH depends primarily on the background signal-to-noise levels. The lower the background s/n ratio of the substrate used for FISH, the better the visualization of the target-probe hybridization. Signal visualization also depends on efficient slide preparation and chromosome fixation. Fixation helps the formation of nucleoprotein cross-links and dehydrates the cells keeping them in their swollen state. Fixation also aids in better attachment of the chromosomes and nuclei to the slides making DNA accessible to the probe[84]. To this end, we tested polymers such as PolyMethyl MethAcrylate (PMMA), PolyCarbonate (PC), Cyclic Olefin Copolymer (COC), and PolyDiMethylSiloxane (PDMS) for their autofluorescence and cell/chromosome fixation. Furthermore, we also developed a microchip with suitable substrate to perform Interphase FISH. The FISH analysis was demonstrated with X chromosome centromeric probe.

3.2. Materials and methods

The following substrates were used for the experiments- PMMA, PC, COC, and PDMS were made by adding base and curing agent in 10:1 ratio (w/w) respectively. The mixture was de-gassed; approximately 1 ml was poured on borofloat glass, and cured at 75 °C for 1 hour. All the PMMA and PC substrates were cut into normal glass slide size (72x 25mm) by CO₂ laser ablation. Borofloat glass slides were used as a control

3.2.1. Chip fabrication

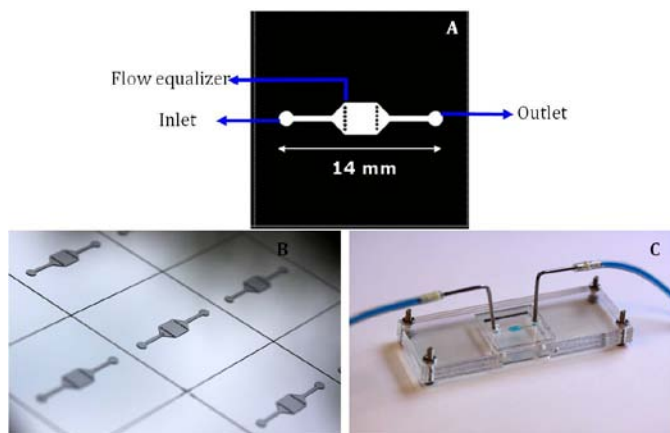


Figure 3.1: A microfluidic chip for FISH assay

- A) Mask design for SU-8 mould
- B) Master for moulding PDMS microFISH device lid fabricated in SU-8 photoresist over silicon wafer
- C) PDMS microFISH device lid moulded using PDMS elastomer.

The master for the FISH device lid was prepared in a cleanroom using a traditional photolithography process (Figure 3.1B). The structures were created on a Silicon wafer in SU-8 photoresist using negative patterning. The recipe for creating the master is shown in (Table 5.1). A schematic of the chip with dimensions is shown as in Figure 3.1A. This master can be reused. The

lid of the micro FISH device is mould in PDMS using traditional soft lithography techniques [85]. The PDMS was then peeled away from the Silicon/SU-8 master. The final PDMS FISH device is then bonded with 20 X 20 mm Pyrex glass. A completely assembled chip is shown in Figure 3.1C.

Fixed cell suspension was passed through the inlets and heated up to 75 °C for 15-20 min. In the following chapter, the effect of cell/chromosome fixation on a closed chip surface is discussed extensively. Some cells were fixed and some flow through the system from inlet to outlet. All the FISH chemicals were passed through the inlet channel. The protocol for FISH chip method was performed according to Vedarethinam and Shah *et al.*, [73]

3.2.2. FISH Sample preparation

Chromosome spreads were prepared by standard methods [86]. Peripheral blood lymphocyte cultures were prepared and treated with 75 mM KCL hypotonic solution for 5 minutes during the harvesting stage. Then fixative (methanol: acetic acid, 3:1) was added to the cells drop by drop. After proper mixing, a drop of the cell suspension was dropped onto a clean slide and it was allowed to air dry. Slides were prepared 2 days before the experiments for chromosome ageing.

3.2.3. Probe preparation and labelling

As commercially designed probes were not used, we designed FISH probes in-house based on the target gene sequence. Probes were made by cloning our unique genomic DNA sequence into Bacterial Artificial Chromosome (BAC) vector. Probe labeling was done by nick translation using Invitrogen Nick Translation System. For labeling, a template containing 150-250 ng/µl DNA in sterile water, Biotin 12 dUTP labeling mix (Roche), and Nick translation mix (Roche) were taken and mixed well. Finally the probe was suspended in

hybridization mix (50% formamide, 10 % dextran sulphate in 2x SSC). The mix was incubated at 37 °C overnight and then stored at 4 °C.

3.2.4. Hybridization:

The slides were treated with RNase (10 µg/µl) at 37 °C for one hour and 2xSSC was used at room temperature for washing. Slides were then dehydrated with increasing percentages of ethanol (70%, 80%, 99%). The chromosomal DNA and probe DNA were denatured at 75 °C for 5 minutes separately. Chromosomal DNA was denatured in 70% formamide placed in a water bath. Slides were dehydrated with ice cold ethanol (70%, 80%, and 99%) and allowed to dry. Labelled probe was directly added to the genomic DNA and the slides sealed to avoid evaporation. The slides were incubated at 37 °C overnight for hybridisation. Post hybridisation wash was done with 50% formamide at 42°C for 15 minutes; final washing was performed with 0.1x SSC at 60 °C and 4xSSC at room temperature

3.2.5. Visualization:

Optimal visualization of the hybridization signal could be achieved by selecting proper fluorescent microscope filters to match the fluorophore wavelength. DAPI (4-6 – diamidine- 2- pheylindole) was applied to the hybridized targets on the slides and mounted with cover slips. A Leica DMRB epifluorescence microscope with senses change coupled camera was used to view and image the hybridization signals.

3.3. Results

3.3.1. Substrate Auto-fluorescence

FISH analysis was performed using PC, COC, and PDMS substrate. The image was taken with Zeiss fluorescent microscope. The image was further

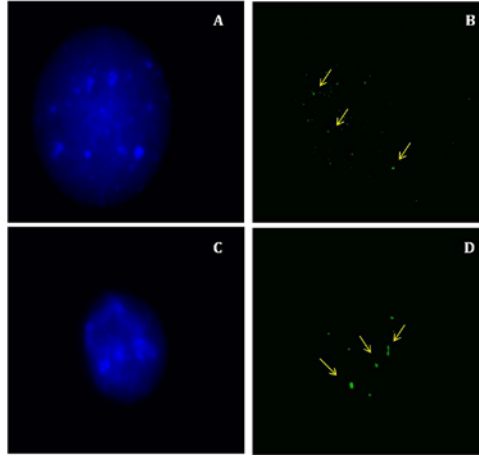


Figure 3.2: FISH assay with COC substrates.

Fluorescence images of the cell were taken after completing a FISH experiment on a glass slide (control) and on a COC slide with overnight hybridization (figures 2A, 2C) respectively. The green dots are FITC signals for non specific probes (figures 2B, 2D). This surface gave acceptable results without auto-fluorescence problems. However, the images taken with the microscope were very foggy.

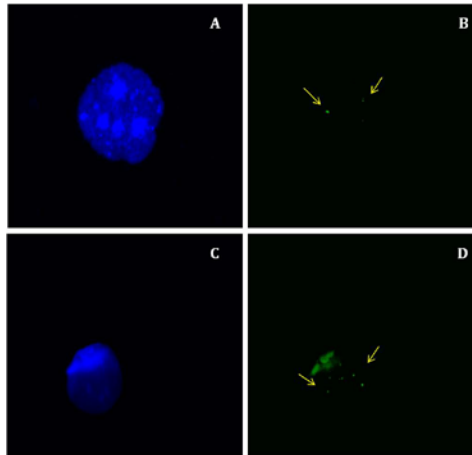


Figure 3.3: FISH assay with PDMS substrate.

Fluorescence images of the cell stained with DAPI and FITC were taken on glass (control) (Figure 2.3A&B) and PDMS slides with overnight hybridization and signals were observed without auto-fluorescence effect (figures 2.3C&D).

observed manually for autofluorescence. The background fluorescence of borafloat glass and other plastics on UV excitation wavelengths determines their applicability for use in miniaturized biological applications. Substrates with the least auto fluorescence are ideal for chip based applications.

Here, we have compared different polymers such as PMMA, PC, COC, and PDMS, to glass to selectively identify the suitable substrate for chip based FISH assay based on their optical properties. As previously known, glass gave better signal visualization with both DAPI and FITC excitation wavelengths

owing to its low autofluorescence (Figure 3.2 & Figure 3.3). But the high costs and fabrication difficulties associated with the use of glass for miniaturized assays turned our focus on the use of low cost polymers. In our experiment cells are fixed in fixative (Methonal: Acetic acid; 3:1). PMMA is not resistant to those solvents. Cracks are observed in the chip (Data not shown) after the cells are fixed on the surface. Due to this reason, PMMA could not further be used in the experiments.

PC showed a more intense signal with DAPI because of slightly higher s/n ratio compared to that on glass (Not shown). It failed to show any hybridization signal with FITC (excitation wavelength of 494 nm), which might be due to variation in the light emission by PC substrate because of its auto fluorescence. COC showed a reduced signal with DAPI which was hindered by the background noise (Figure 3.2C&D). This could be attributed to the surface sensitivity of COC to pre- and post- hybridization washes during FISH procedure. It gave a low hybridization signal with FITC unlike PC. Surface modification of COC could overcome this limitation.

In general, COC exhibits less auto-fluorescence than polycarbonate. We identified a batch-to-batch variation in COC with inconclusive results in our experiments. PDMS produced a good signal with DAPI which was not affected by background and almost comparable to that of glass (Figure 3.3). It showed a good hybridization signal with FITC as well indicating that it is

an optimal surface with the least auto fluorescence next only to glass for use on chip based biological assays.

3.3.2. Immobilization property

The surface properties of substrates are very important for cell immobilization. Hydrophobic surfaces do not support cell immobilization, they have to be either surface coated with some chemicals or a surface modification is required. Though PDMS has less auto-fluorescence, it is very difficult to fix the cells on the surface. To this end, we have extensively

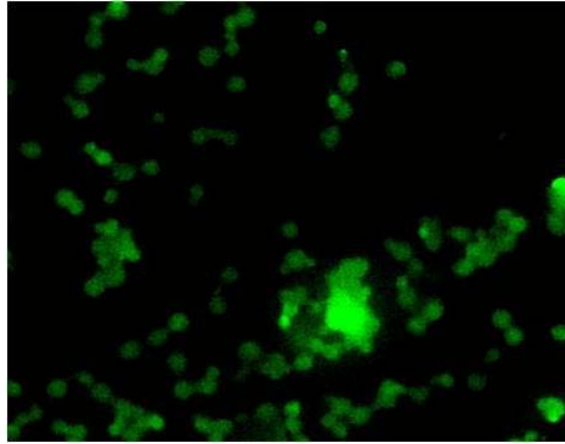


Figure 3.4 : Cells fixation on PDMS chip by temperature treatment.

reviewed for resolving for the fixation issue. If the surface is coated with chemicals such as collagen, can be dehydrated by organic solvents [87]. Nano structures are usually fabricated to enhance cell adhesion using lithography techniques [88], which is complex and expensive. UV treatment or plasma treatment can change the hydrophobic surface property[89] and enhance cell attachment, but it will only last for a few hours. Surface modification processes make the surface highly resistance to organic solvents and other chemicals used in the assay.

In our experiment, fewer amounts of cells were seen attaching on the PDMS surface than on PC and COC. However, by adding a temperature treatment step at 75 °C the evaporation of the fixative is helped and the immobilization strength is also sufficient to perform the process [77]. Many cells were fixed on the PDMS substrate by the temperature treatment (Figure 3.4).

3.3.3. FISH on polymeric chip

FISH signals from the cell nuclei were observed and imaged using fluorescence microscopy. The sample was prepared from patient peripheral blood by standard methods and the probe was fluorescently labeled in house. However,

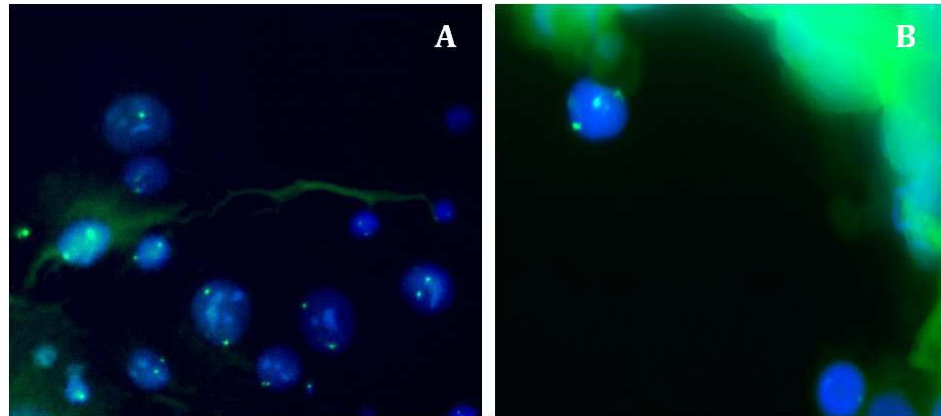


Figure 3.5: Interphase FISH analysis
A) A conventional FISH analysis on glass slide. B) FISH analysis in microfluidic chip. The two green dots highlight the two X chromosomes in the female sample.

the protocol needed to be improved for further optimization of the process. Future work is targeted towards doing FISH on metaphase chromosomes. We have successfully performed interphase FISH analysis on a polymeric microfluidic device with an X chromosome centromeric probe. Two green dots

are representing the successful hybridization of X centromeric probe into chromosome DNA (Figure 3.5A&B). This chip can also be used for other interphase FISH analyses e.g. Multiple Myeloma associated abnormalities. Our final goal is to make a device for metaphase FISH analysis which could not be achieved by this chip due to the fact that rapid evaporation is an important factor in closed chip for achieving metaphase chromosomes. The details on this are described in Chapter4

3.4. Conclusion

We used slide-sized substrates with all the above mentioned polymers to perform FISH on immobilized cells using random probes. We developed a PDMS chip for FISH analysis and successfully demonstrated the complete analysis with X chromosome probe. Our results showed that COC and PDMS were optimal surfaces for a chip based FISH system because of their low auto-fluorescence properties while PC could not be used for this study because of its high auto-fluorescence. Although PDMS had excellent optical properties, we could not immobilize the cells on this surface because of its hydrophobic surface property. However our results were consistent with a previous study [81] regarding non auto-fluorescence properties, which shows PDMS to be a surface of choice for performing lab on a chip FISH analysis. But the challenge with PDMS was the immobilization of cells on this hydrophobic surface. It was achieved by temperature treatment at 75°C for 5 min, but in a closed chip it took longer time to immobilise the cells(See Chapter 4.3.3.). Though we managed to perform interphase FISH on the PDMS chip, we did not get chromosomes in considerable numbers. As PDMS is well known for its gas permeability, this could cause the evaporation of the hybridization mix, with hybridization failure as a consequence. This may explain the low number of FISH signals achieved in the FISH chip. In the future we will investigate the feasibility of achieving chromosome spreads on chip to perform metaphase FISH.

4. Factors Affecting Chromosome Spreads on Polymeric Microfluidic Chip

4.1. Introduction

Cytogenetics is a study of the structural and functional components of the cells, especially chromosomes. Worldwide, 7.9 million infants are born with congenital disorders every year. Many others are diagnosed with acquired haematological diseases. Out of these, some children are diagnosed and treated but others continue to lead a life-time with these birth defects. There are many techniques available for the detection of prenatal and acquired chromosomal anomalies; however, they still exist at very minimal level. In cytogenetic diagnosis, chromosome analysis is performed by routine banding techniques and *in situ* procedures. GTG-banding (G-banding by Trypsin using Giemsa) is a widely used technique, in which metaphase chromosomes are treated with trypsin and stained with Giemsa. GTG banding is useful in detecting structural changes, numerical changes, and any general alteration in the normal chromosomal patterns. The banding technique is not very specific in identifying chromosomal changes in tumour cells and areocentric mouse chromosomes [74] because of smaller genetic alteration. The technological advances of the 1980's have put forward new techniques such as "Fluorescent *in situ* Hybridization (FISH)", which allows for investigation of either specific regions of chromosomes or the whole chromosomes. It is also particularly

useful in analyzing gene expression sites, identifying changes in mRNA synthesis and exploring tumour cytogenetics [90].

4.1.1. FISH Variants

As mentioned earlier, FISH is now a routine cytogenetic technique used in clinical diagnosis. Many variants of FISH have been developed over time. An interphase FISH (I-FISH), is mainly used in diagnosis of haematological anomalies, gene expression analysis, and histone methylation [56, 91]. In I-FISH, samples collected from patients are directly used for FISH analysis without prior culturing of the blood sample. I-FISH can only detect known defects. Metaphase FISH is useful in detecting unknown markers in any chromosomal abnormalities as well as for prenatal diagnosis. It involves analysis of metaphase chromosomes, so the patient blood sample has to be cultured for arresting cells at mitotic metaphase stage.

Multi-colour FISH (M-FISH) is useful to study complex chromosomes rearrangements (CCR) involving two or three joint and break points. In M-FISH, each chromosome is painted with a unique colour, to easily identify rearrangements. Spectral Karyotyping (SKY) is another variant of FISH which requires special software and hardware for Multi-colour image acquisition. Fibre-FISH is yet another FISH variant in which cells are attached on to a slide and DNA/chromatin is stretched using salt/solvents. Highest molecular resolution (approximately 2.3 Kb) can be achieved by Fibre-FISH. However, this resolution is chiefly determined by probe specificity [92].

Generally, in FISH technique, a small DNA fragment called the probe is labeled with a fluorochrome, and allowed to hybridize to a complementary sequence on the genomic DNA. Fluorochromes emit visible light at particular wavelengths, so the hybridization signal can be visualized under fluorescence microscope [61]. Although several techniques are continuously being developed for chromosome analysis such as Array CGH, microarrays, and DNA sequencing [93, 94], classical cytogenetic methods and FISH are still considered to be valuable tools in clinical diagnostics.

4.1.2. FISH- Reagent Requirements and Constraints

Worldwide every year, a minimum of 1 million molecular cytogenetic analyses are performed [92]. Though FISH is one of the most powerful cytogenetic techniques, there are few constraints, which push back its wide-spread utilization in clinical diagnostics. It is laborious, time consuming (3-5 days for analysis including blood culture, sample preparation, slide preparation and assay), and requires skilled technicians to prepare the slides, FISH assay, and image analysis. Culture of blood can be avoided in interphase FISH, but even so the FISH assay itself takes 24-48 hours. Another important issue is cost-per-experiment; mainly the probes and the reagents are very expensive. To overcome these difficulties, a first attempt has been made by Lee *et al.*, [76] to miniaturize the *in situ* platform. Later Sieben *et al.*, developed an improved version of the microfluidic FISH chip to address these issues [42, 77]. Zanardi *et al.*, developed a miniaturized microfluidic chip to address cell immobilization and probe reduction. These papers mainly focused on miniaturizing interphase FISH [95]. Vedarethinam and Shah *et al.*, demonstrated a miniaturized device to create chromosome spreads and perform metaphase FISH [73]; however, much effort is needed to automate the metaphase FISH chip. As an improvement towards automation, it would be ideal to design a device for chromosome spreading inside the chip and then perform FISH analysis. To this end, we investigated various factors affecting chromosome spreading on our miniaturized microfluidic device. If chromosome spreading can be obtained in a closed chamber, that can then be used to carry out a complete FISH protocol on chip [73].

4.1.3. Metaphase FISH and Slide Preparation

There are different protocols for various cell types, such as aminocytes, lymphocytes, solid tumors cells, and leukaemic cells, available for slide preparation, [96]. The traditional way of slide preparation process varies from lab to lab. Metaphase FISH results are completely dependent on the quality and consistency of the well-spread chromosomes. Primarily, the cultured cells

are arrested at metaphase during cell division cycle, followed by a hypotonic solution treatment (0.075 M KCl), which causes cell swelling [84] and aids chromosomes to move from centre towards the periphery of the mitotic cells [97]. After removal of the hypotonic solution by centrifugation, cells are fixed in acetic acid and methanol (1:3) fixative. After few washes with fixative, the cell suspension is prepared at an appropriate concentration and dropped on the glass slide using a Pasteur pipette. Excess amount of the fixative is drained on a tissue paper. The fixative removes the lipids and denatures the protein by dehydration [84, 96] and the cell membrane then becomes more fragile [98]. The fixative also removes the nuclear protein and at the end of the fixation process, chromosomes are obtained with a compact DNA and without the nuclear protein [84]. The slides are allowed to air dry for 2 days, which helps in aging and clear visibility of the chromosomes.

As an alternative to air drying, chemical ageing [84] and dry-heat aging were investigated by Claussen *et al.*, (2002) [97]. Chemical aging showed better FISH signals, as it maintains cell freshness and preserves the chromosome architecture to shorten the hybridization time [96].

Yun Yan Qu *et al.*, developed a chromosome dropper tool and investigated how height and angle affect the chromosome spreading of murine embryonic stem cells[99]. They observed that the chromosome spreads obtained from 0° resulted in the same number of spreads but overlapped, spreads obtained by 30° and 45° on the other hand had a higher number of well-spread chromosomes. Dropping height of 20 cm, 30 cm, and 40 cm yielded better spreads and fewer compact spreads compared to a dropping height of 0.2 cm or 10 cm. They concluded, however, that height may not have much impact on the chromosome spreading but the dropping angle may be crucial. Another important factor to achieve better spreads is water-induced swelling. In contrast, Claussen *et al.*, mentioned that the dropping height does not improve the chromosome spreading but it allows even cell distribution on the slides. After splashing fixed cell suspension, the cells become grainy. At this point, introducing acetic acid and hot steam is critical to get better chromosome spreads.

Moreover, in the presence of a higher humidity (29%), addition of acetic acid followed by water directly on the slides leads to elongation of chromosomes[97]. The methanol in the fixative evaporates faster than the acetic acid and the concentration of the acetic acid on the slide increases. The amount of the fixative differs from slide to slide, which results in variation in the spreading within the same slides [97]. Fast evaporation of fixative from the slides often results in overlapping of the chromosomes and a slow evaporation results in the scattered and tight-spread metaphase chromosomes[100]. Lower humidity settings yield less broken and less scattered chromosomes. For a better analysis, however, the less scattered chromosomes can preferably be used rather than the broken chromosomes. According to Spurbeck *et al.*, 55 % relative humidity and 20 °C would be suitable for aminocytes whereas 60 % relative humidity and 20 °C for lymphocytes.

From literature, we re-examined height and temperature treatment with different time settings in order to implement it in the microfluidic chip. Initially the experiments were performed with glass slides. The result showed that the dropping height is not significant to obtain better spreads supporting Claussen *et al.*, findings [97]. But temperature treatment dramatically increased the chromosome spreading ability. We optimized the temperature to 75 °C for 10 min to yield well-spread chromosomes. A simple, closed microfluidic PDMS chamber with an inlet and an outlet, consisting of a Pyrex glass at the bottom was fabricated to obtain chromosome spreads and perform FISH. Firstly, cold water was passed through the inlet followed by the cell suspension flow and the temperature treatment applied by a hot plate. This chip was unsuccessful in preparation of the chromosome spreads, as no spreads were properly formed. Based on the experiments, we concluded that the basis of the chromosome spreading in the microfluidic device is mainly influenced by an optimum rate of the fixative evaporation. We need to incorporate a mechanism to allow for optimum evaporation of the fixative leading to stretching, flattening, and finally spreading of the chromosomes. This could be achieved by the use of a micro-splashing device. To this end,

we devised a novel splashing device with an open chamber which allows for the optimum evaporation of the fixative. This could in turn be aided by the environmental factors such as temperature of the slide and humidity.

4.2. Experimental methods

4.2.1. Cell Culture and Fixation

Cell suspensions from peripheral blood were used in the experiments. A standard protocol was used for cell culturing [86]. The cells were cultured for 3 days. For chromosome harvesting, colcemid (60 μ l/ml) was added to the cell culture to arrest the cell cycle at metaphase. After 2-4 hrs, hypotonic buffer (0.075 M KCl) was added to the culture at 37 °C for 5-10 min. Hypotonic treatment increases cell volume and disrupts the cell membrane of the red blood cells (allowing their removal). The cells were pelleted and resuspended in 5 ml fixative (3:1 methanol, acetic acid) and transferred to a 15 ml falcon tube followed by few washes with fixative. Finally, the cells were resuspended in 1 ml fixative by drop-wise addition. This suspension was used for further experiments.

4.2.2. Chromosome Spreading by Height Variation

The chromosome spreads were obtained using a variable height. The cells were washed with fresh fixative and resuspended at an appropriate density of cells. The cells were dropped from different heights: 0 mm, where a drop of the fixed cell suspension was placed on a glass slide, 5 mm, 10 mm and 15 mm. Excess amount of the fixative was removed by draining on a wet tissue followed by air drying. The slides were analyzed after overnight air drying.

4.2.3. Chromosome Spreading by Variable Temperature Treatment

Cells were resuspended in a freshly prepared fixative and dropped on a glass slide using Pasteur pipette and excess amount of suspension was removed by draining on wet tissue, immediately the slides were placed on a hot plate at variable temperatures: room temperature (RT), 50°C, and 75°C. The slides were analyzed after 5 and 10 min temperature treatment.

4.2.4. Image Analysis

After obtaining chromosome spreads by variations in the height and the temperature treatment, the spreads were captured using Olympus phase contrast microscope at 20 X magnifications and by Motic camera. Three different splashed spots were analyzed. In each spots 15 random spreads were imaged. The chromosomes overlapping or missing were not scored. Spread area (μm^2) was calculated by Image J software.

4.2.5. Fabrication

Closed chamber

The closed chamber consists of two layers; a bottom layer of a Pyrex glass and a top layer of a PDMS chamber (Figure 4.1 Images of microfluidic device for obtaining chromosome spreadsC). The PDMS chamber was fabricated by a soft lithography method by casting PDMS on a PMMA mould. The PMMA mould was designed and fabricated by micromilling (Folken Glendale, USA) and can be reused several times to fabricate the PDMS chip. Firstly, a silicon elastomer and curing agent are mixed in a 10:1 ratio (Sylgard 184, Dow corning, and USA). The mixture is degassed in a vacuum chamber for 1 hr, to remove bubbles formed during mixing. The mixture is then poured inside the mould and cured at 80 °C for 2 hrs. After curing, the PDMS was peeled off from the mould and inlet and outlet holes were created by inserting a needle with a silicon cartridge. The diameter of inlet and outlet holes was 1.2 mm.

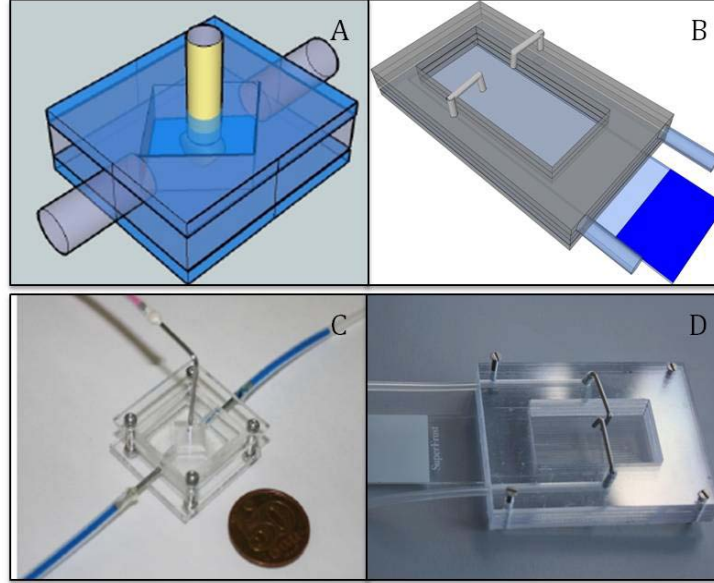


Figure 4.1: Images of microfluidic device for obtaining chromosome spreads
A) Schematic image of closed microfluidic device B) Open splashing device C)
PDMS microfluidic chip bonded with Pyrex glass and PMMA chip holder.
D) PMMA open splashing chip fabricated by milling.

Both layers were bonded together by plasma (Electro-technic product Inc, USA) treatment for one min.

Open chamber

The open splashing chamber is fabricated by adapting a design from Vedarethinam and Shah *et al.*, [73]. The chip consists of four layers (Figure 4.1 B). The bottom layer is used to accommodate a sliding glass slide. Two channels with inserted silicone tubing are milled along the sides of the device to splash ice cold water and the cell suspension separately. At the end of the tubing, two needles (gauge 19) were bent and inserted with tubing to focus splashing spot. All the four layers were screwed together as shown in Figure 4.1D

4.3. Results

4.3.1. Qualitative examination of spreads obtained by height variation

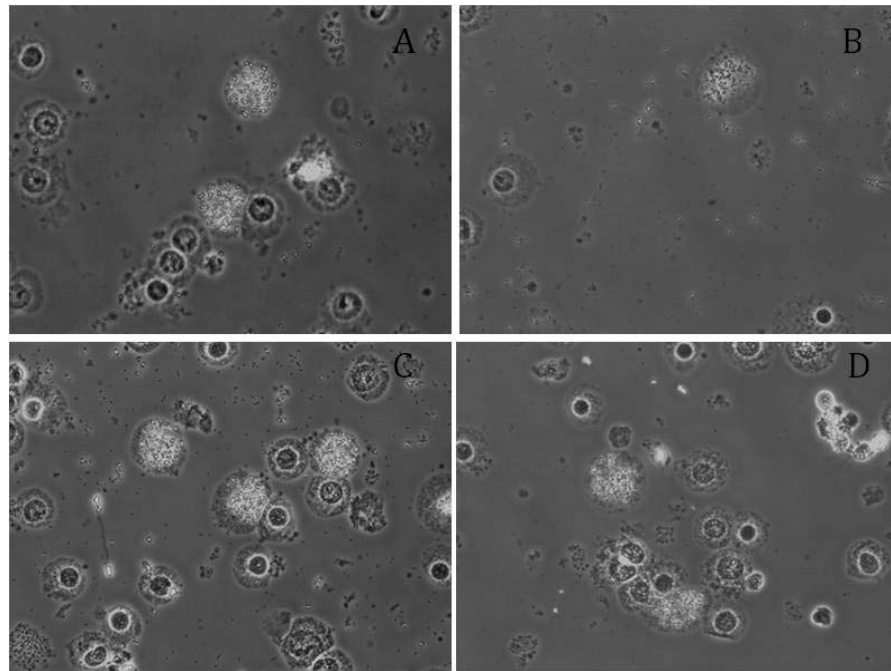


Figure 4.2: Fixed peripheral lymphocyte cells splashed from different heights.
A) 0 mm (a drop placed touching the glass slide) B) 5 mm C) 10 mm D) 15 mm.

Chromosomes were dropped from 0, 5, 10, 15 mm distance onto glass slides and the angle was the same for all these measurements (0°). The spreads were air dried and allowed to age overnight. Afterwards, they were counted and analyzed under phase contrast microscope. We noticed that as soon as the fixed cell suspension is dropped onto the glass slide, the cells were floating in fixative and moving vigorously in all directions. But later the cells touch the surface and are immobilized. Once the fixative starts to dry, slowly the cells are flattened and the chromosome spreads start to appear. Though the cells

were dropped from different heights, we could not observe any significant difference in the chromosome spreading. Regardless of the cell dropping height, cells bombard with each other until the fixative evaporates. The average diameter of the spreads obtained by 0 mm was $1420.57 \mu\text{m}^2$ (Figure 4.3A) and by 20 mm (Figure 4.3D) was $1184.92 \mu\text{m}^2$. Nevertheless our results

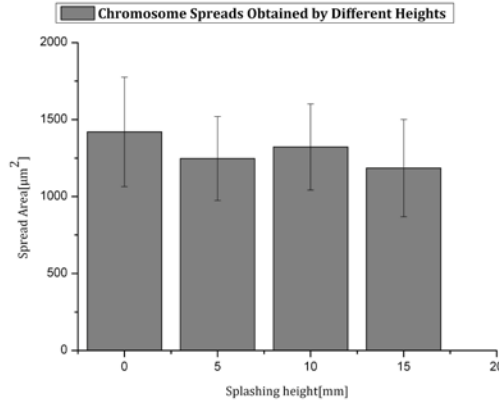


Figure 4.3: Estimated average diameter of spreads by height test

The cell suspension was dropped from different heights in (mm) for obtaining the spreads(μm^2).

show that even at a 0 dropping height, the chromosome spreading can be achieved, concluding that the dropping height has no impact on spreading dynamics.

Evaporation of the fixative is the same, regardless of the height from which the cell suspension was dropped. We therefore concluded that the dropping height does not influence chromosome spreading (Figure 4.2). It may only aid in the even distribution of cells on the slide surface. Dropping may not be of much help in the closed chambers, the cells tend to stop only on the walls of the chamber.

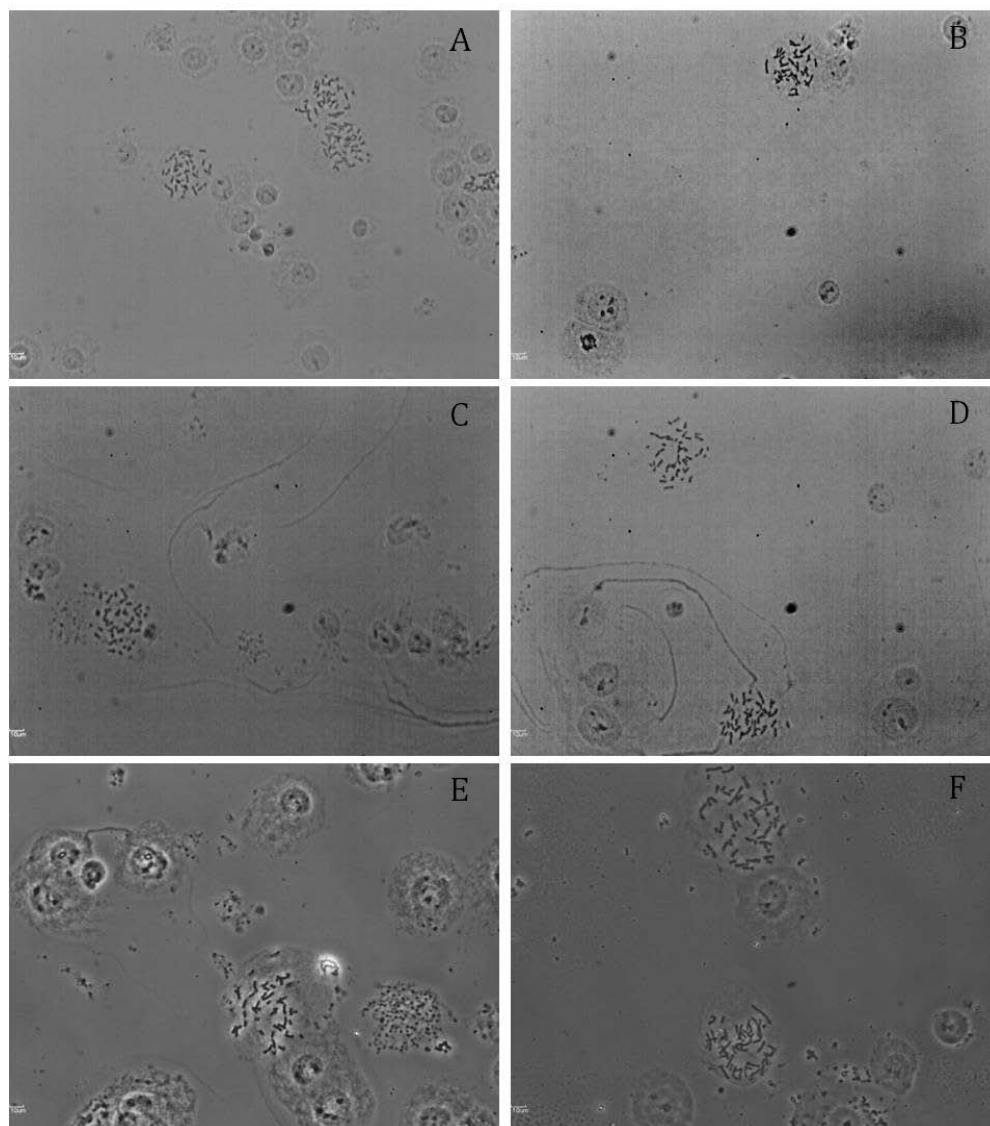


Figure 4.4: Chromosome spreads obtained by different temperature treatment.

The spreads were obtained at 50 °C and 75 °C for exposure time of 5 and 10 min. A) RT after 5 min B) RT after 10 min C) 50 °C after 5 min D) 50 °C after 10 min E) 75 °C after 5 min F) 75 °C after 10 min. Images show differences in chromosome spreads.

4.3.2. Chromosome spreads obtained by temperature treatment

The conventional method of splashing was performed in these experiments with various temperature and time. After splashing, the slides were drained on a wet tissue and transferred on to a hot plate and were heated up at 50 °C, 75 °C or allowed to dry at room temperature (RT). The data was collected at three different temperatures and two different time settings (5 and 10 min intervals) (Figure 4.3). The quality of the chromosome spreads was assessed by evaluating them under a microscope. The results show that temperature improves the spreading but a prolonged temperature treatment (from 5 min to 10 min) does not influence the spreading significantly.

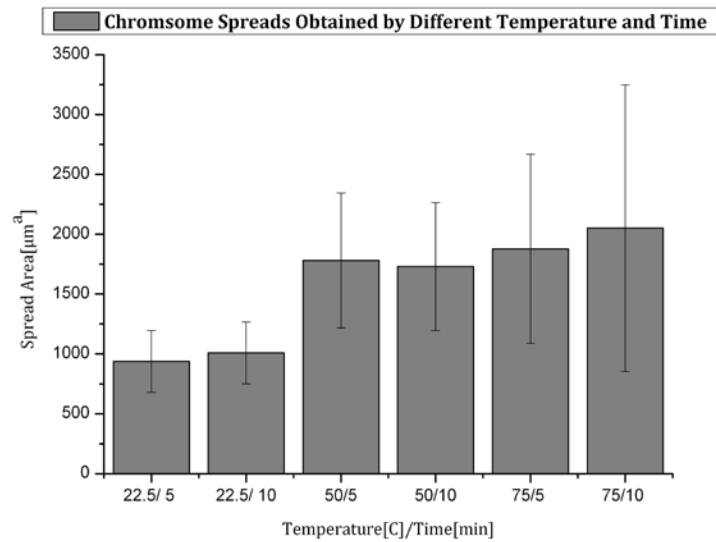


Figure 4.5: Estimated average diameter of the spreads by temperature treatment.

The spreads were estimated diameter (µm²) at different time intervals and temperature treatment.

The average spread area at RT was lower than the other two temperatures. Among these three parameters, our 75° C heat treatment, although this has to be replicated in further experiments (Figure 4.4). When the temperature was increased from RT to 50 °C and 75 °C, the average area of the spreads has also increased. Nonetheless, prolonging the exposure time at these temperatures showed slight improvement at 75 °C treatment. At the same time, it is important not to affect the chromosome architecture with long time temperature treatment.

4.3.3. Key factors for chromosome spreading on a microfluidic chip:

We considered three main factors in the closed FISH chip; dropping height, moistening the surface, and the temperature treatment. In many labs, the slides are stored in ice cold water (3-5 °C), before the slide preparation. Acetic acid in the fixative holds the humidity and evaporates slowly, which helps the chromosomes to spread well. It is widely accepted that moisture on the slide surface is an important factor for the chromosome spreading [97]. Dropping height may influence the even distribution of the cells on the slide; the temperature treatment enhances faster evaporation of the fixative and results in better spreads.

Chromosome spreads with closed chip

The splashing chip shown in (Figure 4.1A) consists of 2 inlets; one inlet for cold water entry and another inlet on the top of the chip to drop the cell suspension. The excess amount of cell suspension will be removed through the outlet. The distance between the top inlet and the surface of the chip is 5 mm. Cold water was passed through the chip followed by splashing the cell suspension through the top inlet. During splashing, the inlet and the outlet were closed in order to allow for the cells to settle. After 5 min, the chip was transferred to a hot plate at 75°C and inlets and outlet were opened to increase evaporation. The chip was observed every 10 min under the microscope and imaged (Figure 4.6B). Initially the cells float in the fixative,

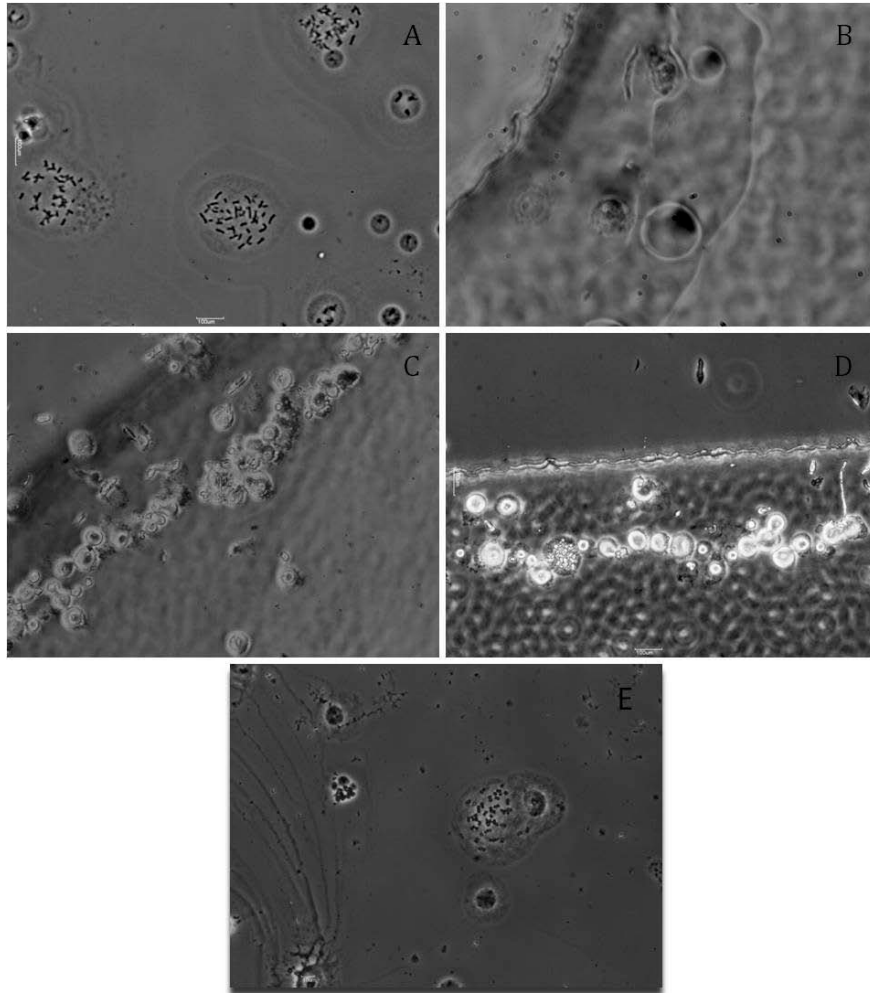


Figure 4.6: Obtaining chromosome spreads in a closed microfluidic device
A) Control slide: the chromosomes were obtained by conventional method.
The spreads were attained at various temperature treatment in a closed micro device B) after 10 min C) after 20 min D) after 30 min E) spreads.

and then get immobilized while the fixative evaporates (Figure 4.6C). In contrast to the traditional method, few chromosome spreads were obtained after 30 min by temperature treatment (Figure 4.6D). Moreover, the number of spreads was much low and the quality of the metaphase spreads was

significantly poorer than those achieved with the traditional methods. It is very difficult to perform FISH with such spreads. The results show that evaporation of the fixative is a much slower process in a closed microfluidic device. Even though we included the dropping, moistening the slide surface, and temperature treatment steps, the poor quality of chromosomes shows that all the above factors are not the only criteria to achieve good quality of chromosome spreads on chip. Instead, we identified rapid fixative evaporation to be a key factor controlling the spread of chromosomes on microfluidic chip.

Polymeric substrates were also tested for chromosome spreading in the experiments (Data not shown). Generally, glass surface is very hydrophilic compared to the polymeric substrate. Adding ice cold water helps to pull the fixative, so that the cells are evenly distributed and the chromosomes spread well. Hydrophobic surface property affects the chromosome spreading by hindering the stretching of chromosomes on the surface, thus resulting in much less scattered and overlapping chromosomes. Nevertheless, results of chromosome spreading on slide-size polymeric substrate demonstrates not only the hydrophobic nature of the surface influencing the spreading of chromosomes, but also the rapid and even evaporation of fixative in a closed chamber.

Chromosome spreads with open chip

To overcome this issue, the open splashing chip was chosen to obtain better results. It is developed by Vedarethinam and Shah *et al.*, [73]. The chromosome spreads can be attained by the open splashing chip, which can rapidly be assembled into a microfluidic FISH device. Double side stencil tape can be bonded on a glass slide, to further perform FISH assay. We developed a similar device (Vedarethinam and Shah *et al.*, [73] (Figure 4.1D) to compare the quality of spreads with conventional methods. We compared the conventional FISH slide preparation method with splashing device; the results acquired by both methods are comparable (Figure 4.7). Nevertheless the spreading area difference varies from slide to slide and within the slide itself [97]. The Splashing device and metaphase FISH chip can be further improved

by incorporating both into one chip for performing FISH. Chromosome spreading dynamics in a closed microfluidic platform has to be further investigated, which will help to complete automation of the chip method.

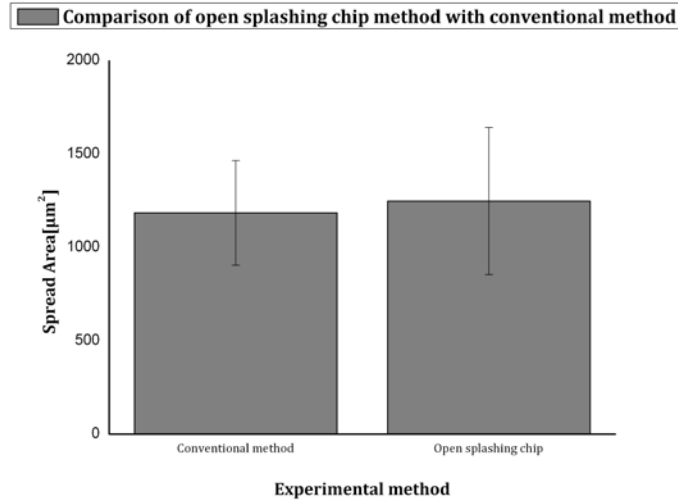


Figure 4.7: Comparison of spreads obtained by conventional and open splashing chip.

4.4. Conclusion

We investigated various factors influencing chromosome spreading in the conventional FISH method based on literature to be replicated in our microfluidic chamber. Our results showed that dropping height is not important for spreading, instead it aids in even cell distribution on the slide. The temperature treatment enhances chromosome spreading. Slides treated at 75°C for 10 min yielded better results. In our closed microfluidic chamber, evaporation of the fixative was a very slow process even with the temperature treatment; metaphase scores were very low and the quality of spreads was very poor. It shows that a rapid evaporation of the fixative is a crucial factor in microfluidic chamber in order to achieve high quality of spreads. To this

end, we developed the novel open chamber as discussed above and compared the quality of metaphase scores between the open chamber microfluidic device and the conventional FISH methods. The results were comparable. The presented open splashing chip is a simple device, but requires further automation to be implemented in a clinical setting after various stages of standardization. However, it can reduce intensive labour, reagent use, and can save the skilled technicians time.

5. Metaphase FISH on a Chip: Miniaturized Microfluidic Device for Fluorescence *in situ* Hybridization³

5.1. Introduction

During the last decade microfluidic techniques have evolved into a new genre of research areas targeting integration of laboratory protocols into miniaturized devices called lab on chip (LOC) or micro total analysis systems (μ tas) [101-105]. The ability to control small sample volumes on micro-sized devices is immensely appealing for techniques involving handling of ultra small volumes of cells or other analytical samples [106]. This has spurred an exponential growth in the number of research articles published in the field of LOC systems targeting complex biological protocols to benefit from the low volume, high throughput and low cost features provided by the microfluidic devices [7, 106-108]. We have created a novel metaphase FISH chip to benefit from the low dead volumes associated with microfluidic devices as FISH protocol reagents and commercial probes are more expensive than gold (over \$400 for 100 μ L of probe used in this work) [42, 77].

³ The material for this chapter is taken from our published article [73]. Vedarethinam, I., Shah, P., Dimaki, M., Tumer, Z., Tommerup, N., and Svendsen, W.E. (2010). Metaphase FISH on a Chip: Miniaturized Microfluidic Device for Fluorescence *in situ* Hybridization. *Sensors* 10, 9831-9846.

FISH is a sensitive diagnostic cytogenetic tool routinely used to visualize numerical and structural chromosomal aberrations [67, 109-116]. The traditional FISH protocol includes steps like immobilization of interphase nuclei or metaphase chromosomes probe labeling, RNase treatment, denaturation of chromosomal and probe DNA, hybridization, post hybridization wash and image processing. FISH is routinely used by cytogeneticists in applications ranging from chromosome labeling and mapping, identification of gene expression sites, tissue analysis, mRNA synthesis tracking, tumour genetic alterations monitoring, identifying infections from viruses and other diagnostic pathological applications including cancer e.g., leukemia [90, 117].

Interphase and Metaphase FISH are two types of commonly used chromosomal FISH techniques. Each has their specific applications and advantages. Interphase FISH is used to identify numerical abnormalities as well as specific structural abnormalities. Thus, Interphase FISH depends on a specific probe, e.g., to detect known translocations, microdeletions or specific chromosomes and hence can only be used to address questions for which DNA probes are available. Lack of conformity of interphase FISH is a major disadvantage when using this technique for prenatal diagnostics [75]. On the other hand, metaphase FISH can be used to visualize the insertion, deletion or other rearrangement involving a specific region of the genome, with a resolution determined by the probe used [113]. Metaphase FISH can be performed on samples with unknown translocations by targeting all the chromosomes using multi-color FISH probes [74], derived from plasmids, cosmids, bacterial artificial chromosomes (BAC) and yeast artificial chromosomes (YAC) [114]. Recently Interphase FISH was demonstrated on a microfluidic device which highlights the obvious benefits of miniaturizing and automating the FISH protocol in a microfluidic system [42, 77, 118]. But metaphase FISH protocol has been elusive owing to the difficulty of handling chromosomes on a chip and fixing the chromosomes on a closed microfluidic device [118]. As a result, in spite of FISH being a very powerful cytogenetic tool, it continues to be an expensive and reasonably time consuming method.

There is clearly a need for replacing the traditional method with a fast and low-cost method making this technique widely available and easy to handle.

Our efforts have been directed at designing a miniaturized protocol for performing metaphase FISH in a controlled manner on a microfluidic device. It is widely known that the results of classical banding techniques, FISH analysis or Comparative Genomic Hybridization (CGH) are dependent on the quality of the metaphase spreads [96]. Hence the result of any FISH analysis depends on consistency of spreading of chromosomes [100]. During the 90s metaphase spreading techniques were widely investigated and many labs developed their own version of optimized standard methods [119-125]. This led to a number of theories on what determines the quality of metaphase spreads on the glass slide, including the distance and the angle of dropping of the fixed cells onto the slide, the diameter of the pipette, the evaporation of the fixative, the temperature of the slide, the hypotonic treatment of the chromosomes, and the whole air drying process [97, 100, 126].

Renewed interest has seen a number of attempts towards making devices for preparation of chromosome spreads, which rely on controlled angle and conveyors [23], temperature gradient and humidity [96] and most recently the chromosome dropper tool which relies on dropping angle and height for fixing metaphase spreads on glass slides [99]. But none of these three devices are remotely close to the miniaturized size we are targeting or give any new insights into the mechanism of spreading compared to what was already published in the 90s. Through literature review and our own preliminary experiments we found that the basis of chromosome spreading is rooted in the optimum rate of evaporation of the fixative from the glass slide.

Hliscs *et al.*, suggests that the mechanism of chromosome spreading is a slow process, which leads to stretching of chromosomes via flattening [98]. Spurbeck *et al.*, mentioned that in the process of spreading the fixative evaporates, leading to build up of surface tension causing the metaphase cell to flatten, which eventually leads to bursting of the cell membrane and spreading of the chromosomes [100]. Henegariu *et al.*, also concluded that

dropping of chromosomes from a height doesn't improve the spreading [96]. Hence, we concluded that in order to realize a micro-splashing device which produces reliable metaphase spreads on a glass slide sufficient for conducting routine FISH analysis; we need to incorporate a mechanism to allow for optimum evaporation of the fixative leading to stretching of the chromosomes and flattening of the cells. This could be aided by environmental factors like temperature of the slide and humidity but the focus has to be on maintaining an optimum rate of fixative evaporation.

In order to account for that, we devised a novel splashing device with open chamber, which allows for easy evaporation of the fixative. The device provides 11 mm dropping height with two inlets—one for cold water and one for the fixed mitotic cells suspension. Apart from the splashing device, a novel metaphase FISH protocol was developed using a microFISH device. This microFISH device provides the possibility of replacing the traditional Coupling Jar and turning it into a miniaturized microfluidic device. We have looked into possible replacements of glass slides with various common polymers but found them unfavorable for FISH protocol on mainly three counts. Firstly, the spreading of the metaphase chromosomes on the glass slides is highly aided by the surface properties of the glass like contact angle and free surface energy.

Secondly, polymers exhibit auto-fluorescent behaviour which hinders the analysis due to interference with the FISH probe signals. Finally, some polymers like PMMA couldn't withstand the fixative used for fixing the metaphase chromosomes and cracked on contact. Hence, we found it ideal to continue using the traditional microscope glass slides as substrate which also offers the benefits of easy integration into existing workplace protocols in cytogenetic labs. In order to minimize the time of fabrication of the microFISH device, an easy protocol for rapid bonding based assembly has been developed. With this the glass slide can be turned into a microFISH device in a matter of seconds by using an adhesive tape-based stencil and bonding technique (described later in Fabrication section). The ease of operation and handling in this protocol has been targeted to allow non-

technical personnel to easily conduct these tests. The rapid fabrication protocol leaves room for conducting multiple tests simultaneously. In addition, the novel protocol allows for an over 20-fold reduction in reagent volume, which is significant considering the costs of commercially available probes. We hope that this first demonstration of metaphase FISH on a chip, will spur renewed efforts in automating metaphase FISH on a chip leading to wider access to low cost, reliable and fast genetic diagnostics in clinical environments. Although the presented device has been developed for metaphase FISH, we feel that the protocol could also be applied for Interphase FISH.

5.2. Experimental Section

Apparatus

A micromilling machine (Folken Industries, Glendale, USA) was used for milling the splashing device, 50 W CO₂ Laser Machine (Synrad Inc., USA) was used for ablating the adhesive tapes, a photolithographic spinner and aligner from Carl-Zeiss were used for fabricating the master mould for PDMS microFISH device and a Zeiss Axio Observer Z1 Fluorescent microscope was used for analysis of the spreads and FISH signals.

Fabrication

The fabrication protocol has been summarized in Figure 5.1. The figure details the protocol for splashing metaphase spreads on the glass slide using the splashing device followed by the rapid fabrication of the microFISH device to perform the metaphase FISH analysis protocol. The details of the fabrication protocol will be explained in the following subsections.

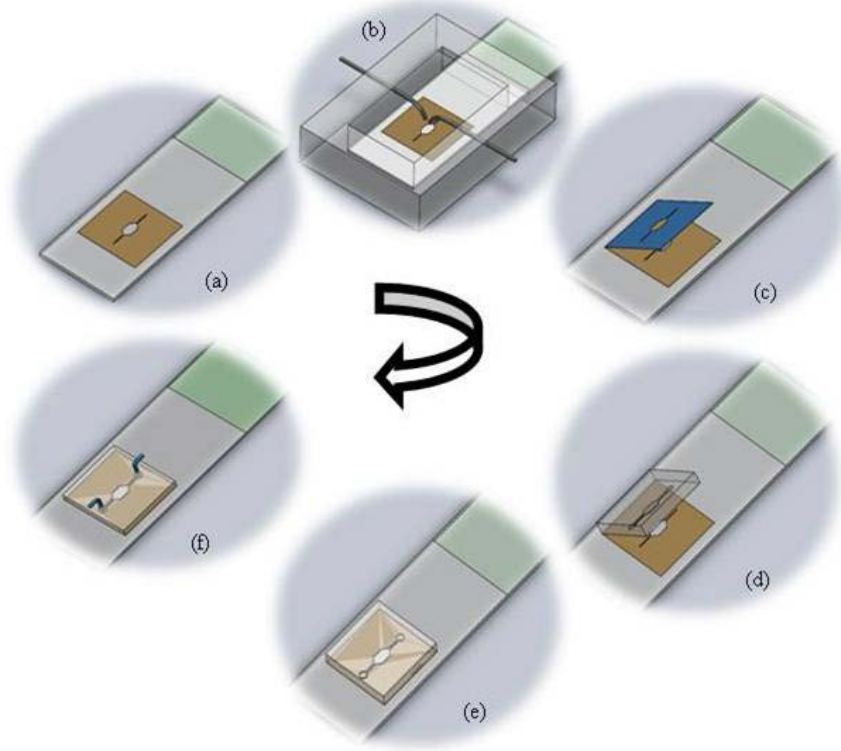


Figure 5.1: Schematic protocol for splashing the metaphase spreads followed by rapid assembly of the microFISH device. (a) Bonding of the double-sided adhesive tape stencil on the glass slide; (b) Spreading of the metaphase spreads in the splashing device; (c) Peeling off the top cover of the double-sided tape stencil to leave only the spreads in the centre of the glass slide and to expose the adhesive layer to bond the PDMS microFISH lid; (d) Aligning the PDMS lid on to the adhesive tape; (e) Assembly of the microFISH by bonding the PDMS lid on to the tape using gentle pressure; (f) Making the interconnection holes and connecting the syringes for world-to-microFISH device fluidic connection.

Glass Slide with Stencil

The stencil for localization of metaphase spreads is created using a double-sided medical grade tape. The double-sided adhesive tape is fabricated using a laser ablation process with a CO₂ laser [127-129]. The laser is operated at 20 W power at 250 mm/s laser velocity using resolution of 800. The operating parameters are set using the Winmark software (Synrad Inc, USA). The design of the tape corresponds to the design of the microFISH device (Figure 5.2). When the tape is ablated using the CO₂ laser, one side of the tape cover is peeled and the tape is bonded to the centre of the glass slide as shown in Figure 5.2. This tape-glass slide complex acts as a stencil when the glass slide is used in the splashing chip to create metaphase spreads. The localization of the metaphase spreads is achieved by removing the top cover of the tape which allows selective patterning on the glass slide in the centre where the tape is fully ablated using the CO₂ laser for creating the microFISH chamber.



Figure 5.2: Glass slide with laser ablated tape stencil.

Splashing Device

The splashing device is fabricated in two PMMA layers by a micro-milling process. The bottom part contains the sliding chamber for glass slide insertion

and the top lid contains the open chamber for evaporation of the fixative. It also contains the two inlet ports for fixed chromosomes and cold water (Figure 5.3). The syringes are slightly bent towards the end to focus the

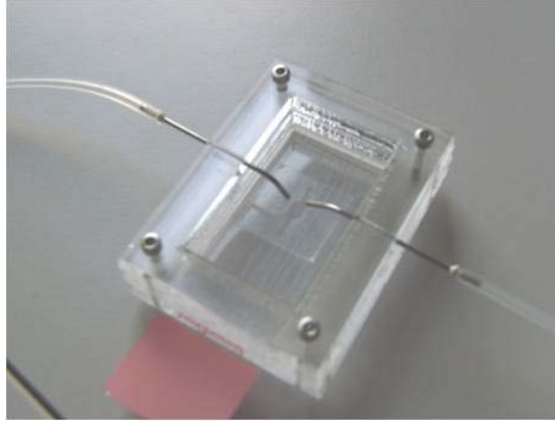


Figure 5.3: The splashing device fabricated with two layers of PMMA. The glass slide with tape stencil is inserted into the sliding chamber in the lower PMMA and the top PMMA plate has a centre open chamber for fixative evaporation and two inlets for glacial water and fixed mitotic cell suspension.

water and chromosome suspension on to the centre of the stencil which exposes the glass slide. The two PMMA layers are either bonded together using thermal bonding [129] or screwed together as shown in Figure 5.3

Master for PDMS Chip and Moulding of the PDMS MicroFISH Chip

The master for the microFISH device lid was prepared in a cleanroom using a traditional photolithography process. The structures were created on a Silicon wafer in SU-8 photoresist using negative patterning. The recipe for creating the master is shown in Table 1.

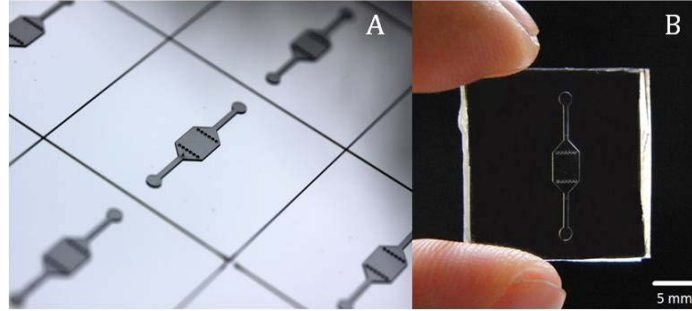


Figure 5.4: Microfluidic device and lid

(A) Master for moulding PDMS microFISH device lid fabricated in SU-8 photoresist over silicon wafer; (B) PDMS microFISH device lid moulded using PDMS elastomer.

This master can be used several times for moulding the microFISH device lid (Figure 5.4A). The microFISH device lid is moulded in PDMS using traditional soft lithography techniques described in [85] and peeled away from the Silicon/SU-8 master. The final PDMS microFISH device lid is shown in Figure 5.4B. The lid is then bonded on the glass slide with metaphase spreads for assembling the microFISH device.

Assembly of the MicroFISH Device

Table 5.1: Recipe for fabrication of SU-8 mould using negative photolithography process.

Step	Type	Parameters
1	Spin-coating of SU-8	Acceleration: 200 rpm/s, Speed: 1,000 rpm, Time 40 seconds
2	Soft Bake	Temperature: 50 °C, Time: 5 hrs, Ramp up Time: 15 min
3	Exposure	Time: 30 seconds Type: Soft
4	Post Exposure Bake	Temperature: 50 °C, Time: 5 hrs, Ramp up Time: 15 mins

The double-sided adhesive tape stencil used for spreading chromosomes is now used as a bonding layer for assembling the microFISH device. Peeling the top cover off the tape provides a silicone bonding layer for the microFISH device lid (Figure 5.5A). The PDMS lid is irreversibly attached to the glass slide using the silicone adhesive layer by gently applying pressure across the PDMS lid (Figure 5.5A and B). Once the device is assembled the holes are made in the PDMS lid for access ports to create interconnection.

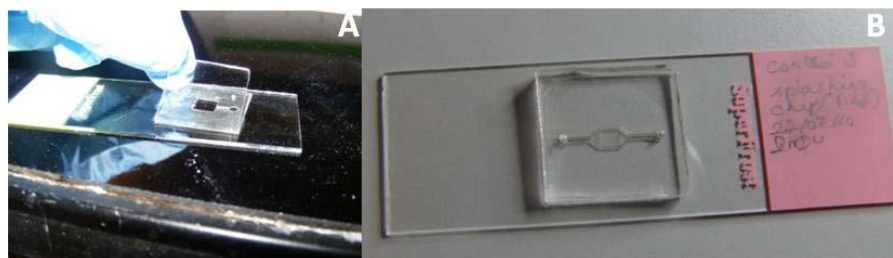


Figure 5.5: Assembling of microFISH chip.

(A) Peeling off the top cover of the double-sided tape stencil to expose the silicone adhesive layer; (B) Assembled microFISH device by bonding the microFISH device lid on to the silicone adhesive layer.

Interconnects

The access holes in the microFISH device lid are made on the top side in the PDMS lid. The holes are made using a 22 gauge syringe needle (0.7 mm outer diameter). The interconnects are formed by inserting an 18 gauge syringe (1.2 mm outer diameter) with a bigger outer diameter than the interconnection holes, which forms a tight seal into the microFISH device. The syringes are connected to silicone tubing with inner diameter 1 mm and outer diameter 3 mm to provide the world-to-microFISH device contacts (Figure 5.6). In order to test the device, the silicone tubings are connected with syringe pumps (not shown in the figure) which provides the pressure needed to actuate the reagents and probes through the microFISH device.



Figure 5.6: Fully assembled microFISH device with interconnects and tubings to connect it to the syringe pump

5.2.1. Procedures

Splashing Protocol

The chromosome suspensions used for creating the metaphase spreads were prepared by standard methods (22). Peripheral blood lymphocyte cultures were prepared and treated with 75 mM KCl hypotonic solution for 5 minutes during the harvesting stage. Claussen *et al.*,. Signified the importance of

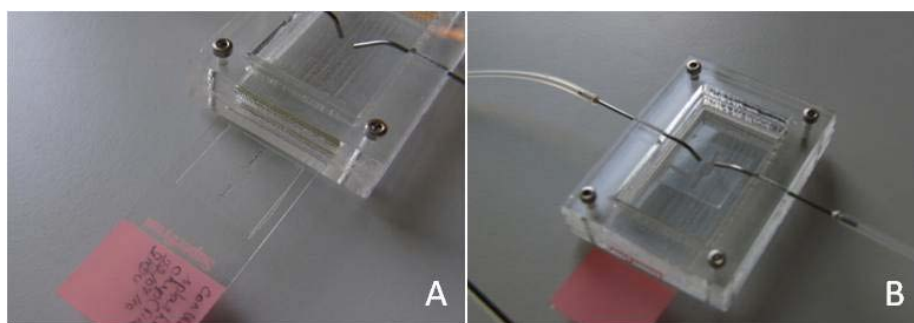


Figure 5.7: Splashing device for obtaining chromosome spreads. A) Sliding of the glass slide with stencil into the splashing device. (B) Splashing device with glass slide. The stencil is positioned under the syringes for splashing glacial water followed by fixed mitotic cells for spreading.

proper hypotonic treatment for achieving good quality metaphase spreads (31). The hypotonic treatment causes the mitotic cells to swell leading to the chromosomes moving to more peripheral locations, which allows for evenly spreading of the chromosomes during fixative evaporation. After the mitotic cells are treated with the hypotonic solution they are fixed using the fixative (methanol/acetic acid, 3:1). The fixative is added drop by drop to the cells all the while mixing thoroughly on a vortex. To compare the spreads achieved using the traditional dropping method and the splashing device, control slides were prepared using the manual dropping method and test slides were prepared using the splashing device. Before dropping the cell suspension on the control slides, the slides are kept in distilled water at 4 °C. On the other hand, the test slides were prepared using the splashing device, where one drop of glacial water was dropped on to the slide followed by a drop of the fixed mitotic cell suspension through the two dedicated inlets (Figure 5.7). The slides were allowed to air dry in the open chamber of the splashing device. As a final step, both test and control slides were heated at 75 °C degrees for 3 min, which improves the fixation of the chromosomes to the slide.

FISH Protocol

The FISH protocol was conducted on both the control and test slides using the microFISH device. After assembling the microFISH device using the glass slides with chromosome spreads, the optimized FISH protocol was conducted on all the slides. The inlet of the microFISH device was connected with a syringe pump for treating the metaphase spreads with the FISH reagents and probes. The specific temperatures associated with the FISH protocol were provided by a hot plate setup. Firstly, RNase (10 µg/µL) was delivered through the inlet. Following the RNase treatment, the microFISH device was kept in the humidity chamber at 37 °C for one hour. Later $2 \times$ SSC was loaded at room temperature for washing the metaphase spreads. The microFISH device was then dehydrated with increasing percentages of ethanol (70%, 80% and 99%), and the device was heated at 75 °C to denature the chromosomal DNA for 5 minutes. Simultaneously, probe DNA was denatured at 75 °C for 5 minutes in the water bath. Then the probe was entered into the

Table 5.2: Comparison of conventional FISH method and microFISH protocol showing over 23 fold reduction in total reagents used and a 2 fold reduction in the probe volume (highlighted in yellow) which is the most expensive reagent for conducting FISH analysis. All steps of the FISH protocol are presented and in case of the microFISH protocol the flow rates used for the FISH reagents are also presented.

Step No.	Name of the step	Conventional protocol	microFISH protocol	
		Vol	Vol	Flow rate
1	RNAse	100 μ L	25 μ L	5 μ L/min
2	Wait 60 mins			No flow
3	Wash 2 \times SSC	75 mL	3 mL	200 μ L/min
4	70% Alcohol	25 mL	1 mL	200 μ L/min
5	90% Alcohol	1 mL	1 mL	200 μ L/min
6	100% Alcohol	1 mL	1 mL	200 μ L/min
7	Drying-Air			NA
8	70% formamide	25 mL		NA
9	90% Alcohol	25 mL		NA
10	100% Alcohol	25 mL		NA
11	Probe	10 μ L	5 μ L	2 μ L/min
12	Hybridisation time	Overnight	Overnight	No flow
13	50% Formamide	75 mL	3 mL	200 μ L/min
14	0.1 \times SSC	25 mL	3 mL	200 μ L/min
15	4 \times SSC	25 mL	1 mL	100 μ L/min
16	1 \times PBS	25 mL	1 mL	200 μ L/min
17	DAPI	15 μ L	5 μ L	2 μ L/min
Total Reagent volume		327.2 mL	14.1 mL	

device and the inlet and outlet were closed to avoid evaporation of the probe. The microFISH device was incubated in the humidity chamber at 37 °C

overnight for hybridization. Subsequently, 50% formamide was delivered as post hybridization wash at 42 °C. Final washing was performed with $0.1 \times$ SSC at 60 °C, $4 \times$ SSC and $1 \times$ PBS at room temperature. The details of the conventional FISH protocol and optimized FISH protocol can be found in Table 2. DAPI was applied to the hybridized targets on the test slides in microFISH chamber and in case of control samples, slides were mounted with cover slips. The Zeiss AxioObserver Z1 fluorescent microscope was used to view and analyze the samples for counting the metaphase spreads and the FISH hybridization signals. The chromosomes spreads on the control and test slides were counted manually using the traditional method.

5.3. Results and Discussion

5.3.1. Splashing Device and Metaphase Spreads

The motivation behind the splashing device was to create a microfluidic device which can provide reliable and sufficient number of metaphase spreads on a glass slide. After spreading the chromosomes on the slide using the

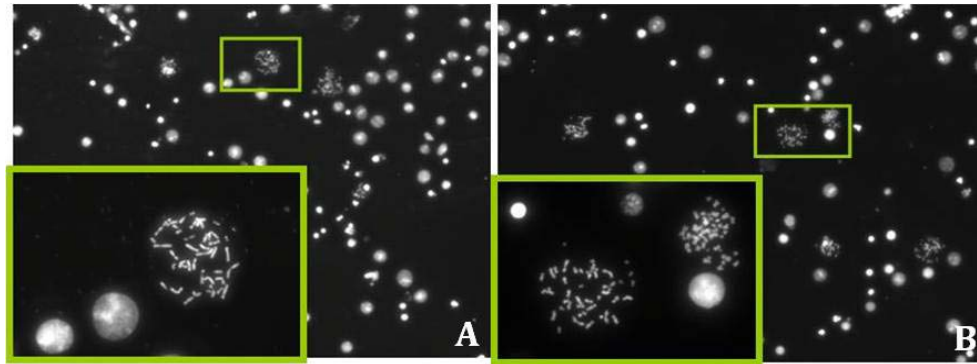


Figure 5.8: Metaphase spreads obtained by splashing device
 A) Metaphase spreads stained with DAPI on the control slides prepared using the traditional dropping method (Inset—40 \times resolution); B) Metaphase spreads stained with DAPI on the test slides prepared using splashing protocol (Inset—40 \times resolution).

traditional method and the splashing device, the slides were stained with DAPI. The metaphase spreads were counted manually in the fluorescence microscope at $20\times$ magnification. Figure 5.8A and B show the images of the metaphase spreads obtained using the conventional method and splashing device respectively. In order to validate the splashing device protocol, we conducted tests using two different cell suspension samples. Table 3 shows the comparison of average spreads obtained using the two techniques.

Even though the average numbers of spreads obtained by using the splashing device are comparatively lower, we could obtain sufficiently good chromosome spreads in order to perform the FISH analysis. The lower chromosome spread counts may be due to the splashing conditions such as height, temperature

Table 5.3: Average number of metaphase spreads found in the microFISH chamber (3×3 mm²) using manual dripping method and our splashing device protocol.

	Sample 1	Sample 2
Control Slide	41	52
Splashing device	29	34

and humidity as described in the introduction. While in average the number of chromosome spreads was significantly lower in the splashing device, it must be noted that in certain samples, we found the number of chromosome spreads to be higher compared to the control slides. Considering that the splashing experiments were not studied in detail, we believe that the splashing protocol can be optimized by altering and controlling the various other factors like temperature, humidity, etc.

Moreover, the simple fabrication protocol of the splashing device using micro milling of PMMA sheets allows us to control the height of splashing if needed by addition of more PMMA layers to increase the height of the splashing syringe. The integrated stencil and bonding layer of the adhesive tape allows for localization of the metaphase spreads on the glass slide in the splashing

device. Thus, the splashing device not only provides an ability to reliably create metaphase spreads on glass slide but also provides a possibility of automating the image analysis procedure in the future due to ease of finding the metaphase spread using software and an automated stage. A number of companies (Zeiss, BioView and Vysis) and research groups are working on optimizing the data acquisition protocols as this continues to be a major issue after the FISH protocol [130, 131]. Automation of the analysis procedure is currently an on-going work where we are aiming to divide the microFISH device chamber into smaller segments to create smaller FISH microchambers corresponding to the microscope objective.

5.3.2. FISH Protocol Results

After the preparation of the chromosome spreads, FISH analysis was performed on the peripheral blood lymphocyte chromosomes using an X chromosome centromeric probe. A female patient sample was used in order to show the validation of the microFISH device protocol and the results obtained with XX chromosomes (Figure 5.9B) confirm the successful FISH analysis

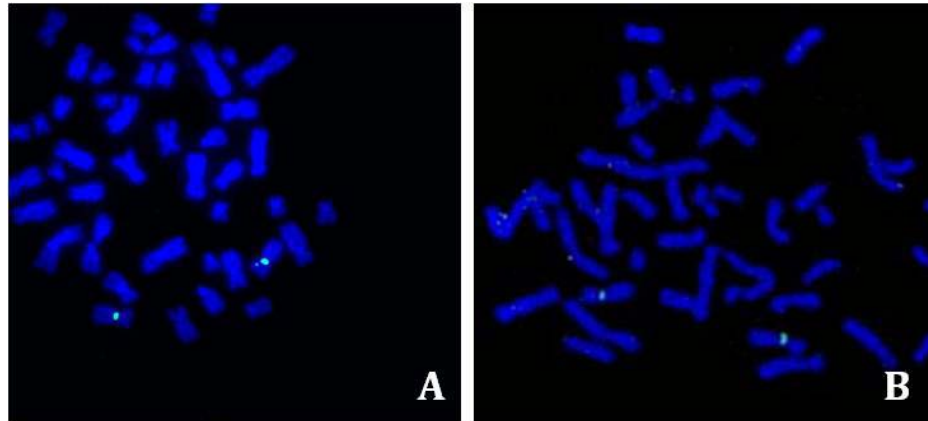


Figure 5.9: FISH analyses performed by microfluidic device

A) FISH analysis on the control slide. The two green dots highlight the two X chromosomes in the female sample; B) FISH analysis on the test slide. The two green dots highlight the two X chromosomes in the female sample.

result. The conventional FISH protocol was also performed on the slides to compare and evaluate the microFISH device efficiency (Figure 5.9B). As can be seen in Table 2, the reagent volume needed in traditional FISH analysis is 327 mL, but now has been reduced to only 14 mL, which is a major step in reducing the costs associated with FISH analysis. As mentioned earlier, the most expensive reagent in traditional FISH analysis is the probe (costing approx. \$90 per test). In the microFISH protocol the volume of this has been reduced to half (highlighted in yellow in the Table 2). This alone cuts the cost associated with conducting the routine FISH analysis in half. Considering the cost of fabricating these microFISH devices is less than \$2, these successful FISH results will give a major push towards making genetic analysis a routine screening test.

5.4. Conclusions

We have successfully demonstrated the first metaphase FISH on a microfluidic device. This work also presents an alternative method for slide preparation and hybridization of FISH probes. The achievements gained in the present study will be used in further improvement of the methodology and aim to develop a completely automated system for performing miniaturized FISH on chip. The micro splashing device designed for spreading metaphase chromosomes on a glass slide provides more reliable and easy alternative for creation of metaphase spreads. The rapid and easy assembly protocol for the microFISH device allows for quick transformation of a simple glass slide into a microFISH device, which makes it an ideal solution for integration into existing work routines at cytogenetic labs. Our current efforts are focused on further miniaturization of this device, which will offer significant benefits with respect to sample preparation and reduction in reagent costs. We are also working towards improving the chromosome spreading protocol by further miniaturization of the splashing chamber and localized micro-spotting of fixed cells, optimizing the hybridization efficiency using temperature and electro-kinetic effects, automating the protocol by incorporation of reagent reservoirs and improving the analysis by developing

automated software for data acquisition and metaphase spread recognition. In the longer run, we envisage to create a chromosome total analysis system providing a more efficient and cheaper solution to the traditional protocol and enable faster diagnosis.

6. A Closed Metaphase FISH Chip; towards Semi-automation of FISH Chip

6.1. Introduction

In the previous chapters, a successful miniaturization of the metaphase FISH technique was presented. In this chapter we will present an effort to optimize the protocol and automate the FISH assay. Most of the standard biological assays involve pre- and post washes for 15-20 min with short intervals. This process is labour intensive and wastes the skilled technicians' time [57, 58]. In lab-on-a-chip technology, we can incorporate the reagent sampling, extraction, filtration, and detection, into one closed cartridge. This could be used in point of care diagnosis for rapid, low cost diagnosis without the need for skilled personnel and large laboratory instruments.

In the approach we follow the reagents are filled inside cartridges and are separated from each other by air bubbles. They can be stored for longer time without getting mixed in the cartridges. This can be implemented in FISH chip, provided a single chip is designed to obtain well-spread metaphase chromosomes followed by FISH analysis. It is clearly understood that rapid evaporation of fixative is a prerequisite for chromosome spreading. To this end and based on the results of the previous chapter, we designed a single chip, where chromosome spreads are obtained and FISH can be performed. A two layered COP polymeric chip was fabricated by micro milling. The top

layer is a chamber with perforation holes at the top of the chamber. It facilitates evaporation, so during the FISH analysis the perforated area will be

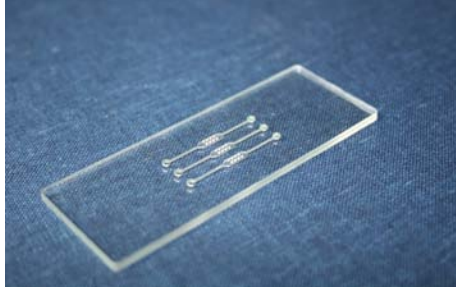


Figure 6.1: A closed single polymeric chip for FISH analysis.

sealed with PCR tape. Here, we present our preliminary results of single polymeric chip for chromosome spreading and cartridges for storing chemicals.

6.2. Materials and methods

6.2.1. Fabrication

The chip consists of two layers, fabricated with COP substrate. The top layer has the milled chamber, where all the reactions will take place. The chamber has 0.5 mm diameter perforations to facilitate evaporation of fixative. It was thermally bonded together with another COP layer, where the chromosomes are to be fixed (Figure 6.1).

6.2.2. Spreading

The fixed cell suspension was introduced inside the chamber with inlet connected with silicon tubing. It allows for the cells to settle at the bottom of the chamber. The chip will then be transferred to hot plate and heated up to 75 °C for 5 min.

6.2.3. Loading FISH reagent in cartridges

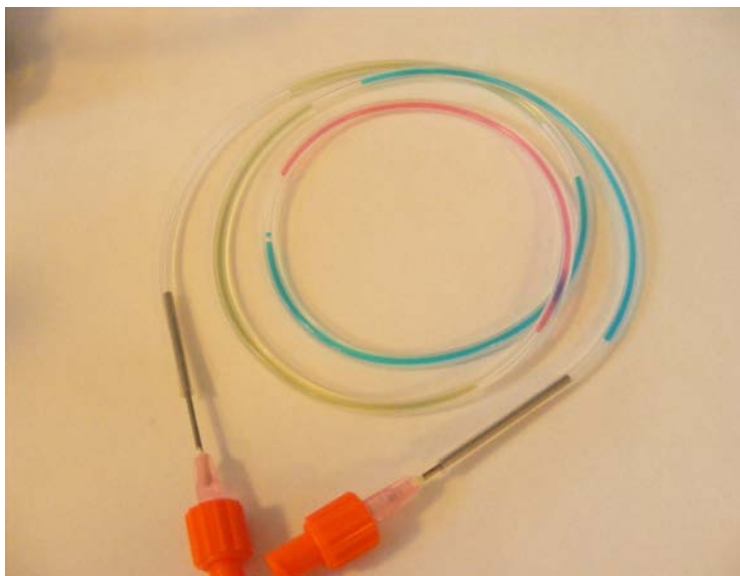


Figure 6.2: A demonstration for loaded reagents with air-spacer by filling different color dyes.

Polyethylene cartridges (tubing) were used to store the FISH chemicals. A syringe pump was used to load and deliver the chemical reagents inside the tubing. All the chemical reagents were filled by withdrawal of liquids and distinguishing from each other with 50 μ l air –spacer (Figure 6.2). This air-spacer facilitates drying of the slides as well. The probe was delivered by pipette and can also be stored in the tubing. The reagents used in the FISH analyses are shown in table 3. The FISH protocol can be obtained from Chapter 5.2.1.

6.3. Results and Conclusion:

The chromosome spreads obtained by our closed chip were comparable to those of the conventional method. More number of spreads was observed in the chip (Figure 6.2) relatively more number of metaphase plates was counted. This chromosome spreading can further be improved by variations in time and temperature exposures. With the obtained chromosomes in closed chip, FISH analysis was carried out. The results did not give any significant

Table 6.1: Comparison of FISH protocol- reduction in reagent volumes between conventional method and chip method.

Step No.:	Process	Traditional FISH		microFISH protocol		
		Vol	Time	Vol	Flow rate	Time
1	RNAse	100 μ L	1 min	25 μ L	5 μ L/min	5 min
2	Wait		60 min		No flow	45 min
3	Wash 2 \times SSC	75 mL	5 min(3 \times)	3 mL	200 μ L/min	15 min
4	70% Alcohol	25 mL	2 min	1 mL	200 μ L/min	5 min
5	90% Alcohol	1 mL	2 min	1 mL	200 μ L/min	5 min
6	100% Alcohol	1 mL	2 min	1 mL	200 μ L/min	5 min
7	Drying-Air		5-10 min		NA	
8	70% formamide	25 mL	5 min		NA	
9	90% Alcohol	25 mL	2 min		NA	
10	100% Alcohol	25 mL	2 min		NA	
11	Probe	10 μ L	20 min	5 μ L	2 μ L/min	
12	Hybridisation time		Overnight		No flow	12 hrs
13	50% Formamide	75 mL	5 min(3 \times)	3 mL	200 μ L/min	15 min
14	0.1 \times SSC	25 mL	5 min(3 \times)	3 mL	200 μ L/min	15 min
15	4 \times SSC	25 mL	5 min	1 mL	200 μ L/min	5 min
16	1 \times PBS	25 mL	5 min	1 mL	100 μ L/min	5 min
17	DAPI	15 μ L	1 min	5 μ L	2 μ L/min	2.5 min
		327.2 mL	14 - 16 hrs	14.1 mL	13 hrs	

FISH signals despite having achieved good metaphase spreads. This indicates that there were some issues with the hybridization process. In cartridge FISH

the chromosomes are fixed on a glass substrate; it conducts and transfers heat, which is a prerequisite for hybridization.

In polymer, thermal conductivity is poorer than in glass with the result that hybridization is hindered. Hot plate is not suitable for DNA denaturation, as

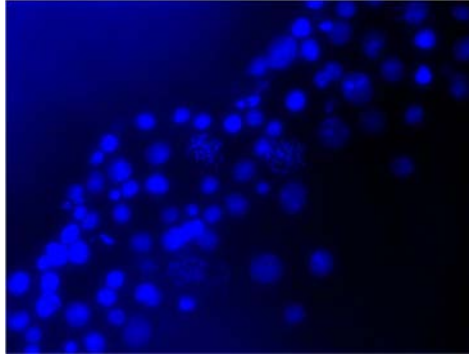


Figure 6.4: Well-spread chromosomes obtained in a closed polymeric chip.
The chromosomes are stained with DAPI.

it does not always provide the right temperature, and the fluctuation is ± 5 %. A hybridization oven would ideally be suitable for DNA hybridization

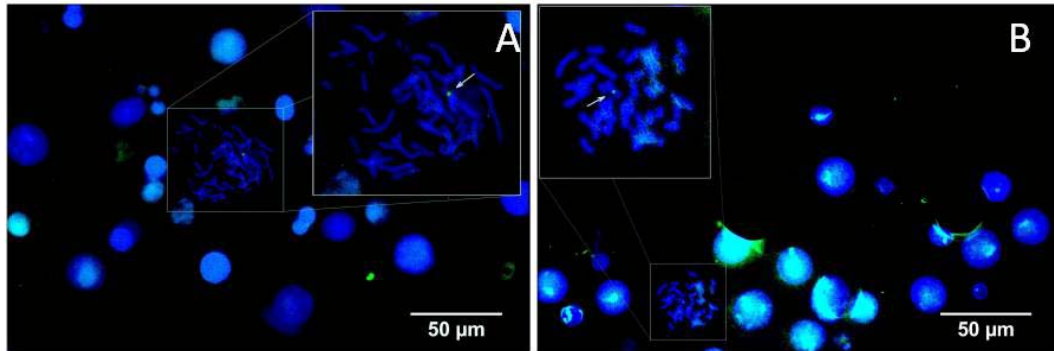


Figure 6.3: FISH analysis in a closed microfluidic chip
A) Conventional metaphase FISH signal (green dot) B) Cartridge FISH signal.

The probe was targeted to X chromosome centromere. Another FISH assay was conducted with reagents loaded into cartridges. The results were examined using a Zeiss Axio Observer Z1 fluorescent microscope. The background S/N ratio was high indicating that the washing step has to be improved. Unbound probes will produce non-specific signals. Only one signal (green dot) suggests that the sample is from male donor (Figure 6.3B). We could observe more interphase signals than metaphase signals. The DNA in interphase cells is loosely packed and like spaghetti, so the probe can easily bind with it. In metaphase the DNA is condensed and tightly packed; therefore it is crucial to denature the DNA at the right temperature followed by treatment with probe. Cartridge FISH shows promising results. However, the hybridization has to be improved.

6.4. Conclusion and outlook

These are preliminary results of the automation process. However, combining the FISH analysis on to a single chip and storing the chemicals inside cartridges can possibly be achieved after few trials. This achievement would be a step towards semi-automation. This system could be redesigned for complete automation by utilizing a programmable syringe pump for required intervals, Peltier heater for temperature control, and special program for signal scoring.

Part II Brain Slice Culturing

7. Introduction to Brain Slice Culturing

7.1. Exploring Cellular Dynamics at Nanoscale (EXCELL)

This work is a part of the EU-funded FP7 project “Exploring Cellular Dynamics at Nanoscale” (EXCELL) which aims at understanding the dynamics of cellular functions and molecular machinery using miniaturized probes down to nanoscale able to address subcellular compartments. In EXCELL, Lab-in-a-Cell (LIC) approach is being developed, making it possible to study the fate of single cells in their natural environment as a part of a cell population in terms of secretory compounds, e.g., neurotransmitters released

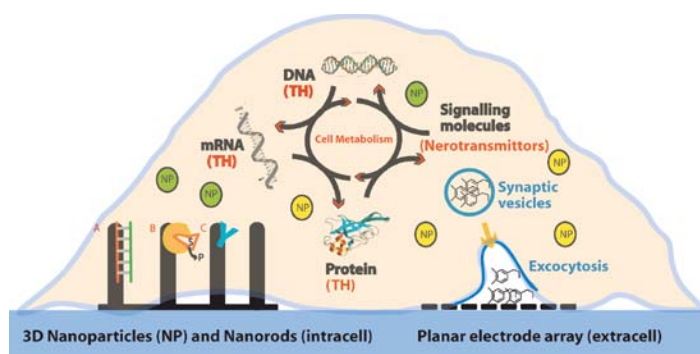


Figure 7.1: A schematic view of the general goal of EXCELL project. Different parameters of cellular dynamics can be studied simultaneously using intra- and extra-cellularly placed electrochemical and optical sensors. (www.excell.nu).

through exocytosis, and cell-to-cell communication.

The approach is based on microfluidic cell/tissue culture systems integrated with 3D (pillar) electrodes. The project involves expertise from cell and molecular biology, neuroscience, as well as micro- and nanotechnology to develop a state-of-the-art technology and experimental procedures to study neuronal stem cells and their interaction with native neurons in brain tissue. This will give a deeper insight into the biological dynamic processes (Figure 8.1), such as gene expression and protein synthesis as well as physiological responses, which can help researchers to develop cell-based therapies for neurodegenerative diseases.

The main focus of the second part of my PhD thesis as a part of EXCELL project has been designing of a transparent polymeric microfluidic array system for long-term brain tissue culturing, schematically shown in Figure 7.2, to facilitate integration of human neuronal stem cells as an approach to develop stem cell therapy for Parkinson's disease. As a continuation of this research, such a system could be equipped with planar (2D) microelectrodes [132, 133] to investigate the differentiation of neuronal stem cells (growth and exocytosis) and 3D microelectrodes [134] for potential recordings from cultured tissue as a response upon stimulation of the integrated stem cells. During the system development two main challenges needed to be addressed:

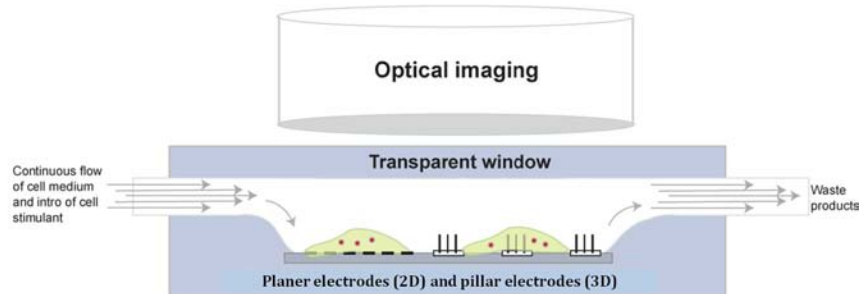


Figure 7.2: A schematic sideview of a transparent microfluidic system comprising planar (2D) and 3D microelectrodes.

i) culturing of brain tissue for at least three weeks under optimal conditions (nutrient and O₂ supply as well as pH and temperature control) in the microfluidic system, and ii) monitoring of stem cell integration and differentiation with a sufficient optical quality.

7.2. Brain tissue cultures

Brain tissue cultures are used in neuroscience to enable the mimicking of *in vivo* conditions in a wide range of applications, including study of pathogenic conditions, neuronal plasticity after injuries, stroke, drug development, therapeutic approaches for treating neurodegenerative diseases, and neurotoxicity [135-137]. In such applications, it is important to maintain proper conditions including gas exchange, nutrient supply, and temperature to preserve the tissue architecture, cellular morphology and synaptic connections. The advantage of *in vitro* cultures is that they can be intervened for experimental purposes without compromising the *in vivo* conditions. Rats and mice are primarily used as animal models for such studies. More recently these have been available as transgenic animals designed to express fluorescent proteins, eg., Green Fluorescent Protein (GFP). This has been achieved by transfecting the animals with viral transfection vectors [138] and screening the offspring for expression of the desired properties [Tonnsen *et al.*, 2007]. For *in vitro* cultures, brain tissue is utilized as slices with thickness ranging from 150-700 μm prepared from animals of the postnatal period p3-p9, i.e. 3-9 days after birth.

During a brain tissue culture assay, it is important to determine the tissue viability and neuronal fate. The commonly used viability assays are (i) propidium iodide intake assay (PI) (ii) lactate dehydrogenase (LDH) assay (iii) metabolic toxicity test (MTT) and (iv) electrophysiological measurements. PI is a fluorescent dye, the application of which is based on cell membrane permeability. It is taken up into the cells if the membrane integrity is compromised, i.e. it stains the nonviable cells. Upon entering the cells, it is integrated into the nucleic acids. The stained cells can be counted

using an epi-fluorescence microscope. LDH is naturally present in cells. In LDH assay, the efflux of the enzyme into culture medium is correlated to cell membrane damage or breakage. A small amount of culture medium is pipetted out from an on-going culture to perform the assay. In MTT, the metabolic function of the cells is assessed by using the dye, which is initially yellow and upon mitochondrial activity is converted to purple. The colour intensity can be directly interpreted as a proper metabolic function of the cells. The measurements take place by colorimetric analysis. It is mainly used for testing cell toxicity. Electrophysiological measurements are very common in neuroscience enabling the assessment of individual neurons. Since staining used in PI and MTT affects the slice viability, these assays can be only performed as a final assessment. In an analogous way, during electrophysiological measurements tissue is kept for considerable time under conditions that are not appropriate for culturing; hence, this technique also serves as an endpoint assessment. Only LDH assay can be used when culturing is ongoing.

7.3. Traditional brain slice culture techniques

The most commonly used conventional brain slice culture methods are (i) roller tube method and (ii) interface method. When using the roller tube method (Figure 7.3 A), brain slices are attached on a glass cover slip with collagen or plasma clot. The cover slip is subsequently placed into a plastic tube containing culture medium and let rotate slowly [136]. The slow rotation ensures oxygenation of brain slices due to continuous change of the liquid-gas interface. Usual culture periods are about 4 weeks, during which the slices undergo thinning from 150 μm to 40 μm . Brain slices cultured in roller tubes are ideal for microscopic detection and provide good access to neurons to conduct electrophysiological measurements. However, the thinning of the tissue during culturing causes great experimental variations [136]. The interface method (Figure 7.3B) is based on stationary culturing. Brain slices are kept at the air-medium interface on a semi-porous membrane over the

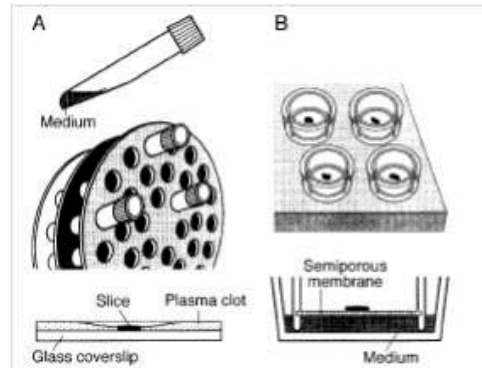


Figure 7.3: An overview of traditional brain slice culture methods.
 A) Roller tube method. B) Interface culture method. Image is
 modified from Gahwiler *et al.*, 1997 [136].

entire culture period (ref). They receive oxygen from above and medium from underneath by capillary action. In contrast to the roller tube method, there is no significant change in the thickness of the slices even during long-term culturing. When using the interface method, brain slices are ideally suited for studying three dimensional tissue sections in, e.g., morphological and biochemical studies that require large amounts of tissue.

Although the traditional culture methods are useful for even long-term culturing of brain tissue, they have limitations in terms of electrophysiological studies since it is difficult to perform tissue culturing and simultaneously record neuronal activity.

7.4. Stem cell therapy for Parkinson's

Parkinson's disease (PD) is a neurodegenerative disease characterized by dysfunction and loss of dopamine releasing (dopaminergic) neurons in substantia nigra par compacta, which innervate the striatum [133]. The disease affects more than 6 million people worldwide and causes slowness of

movement, tremor and muscle stiffness. Other symptoms include sleep difficulties, depression, anxiety, and speech impairments. The neuronal dysfunction and ultimately death can be caused by mitochondrial dysfunction, oxidative stress, inflammation, endogenous / environmental neurotoxins, and genetic predisposition [134]. Levodopa (L-DOPA), a precursor of dopamine synthesis in cells, has been used as a medication for the past decades. It is been reported that patients taking L-DOPA have severe side effects, such as impulse control disorders, compulsive medication intake, and aberrant repetitive disorders [139].

In the late 1980s, fetal tissue implants were adopted to treat PD patients [140]. This process involves transplantation of human fetal ventral mesencephalic portion directly into striatum. Though dopaminergic neurons are identified in the patients after the transplantation, the improvement was minor due to low cell survival. Moreover, the transplanted fetal tissue could not upon differentiation to dopaminergic neurons supply a sufficient amount of dopamine. In addition, the usage of fetal tissue results in ethical and religious controversies.

In a mature brain, interestingly, adult stem cells differentiate into neuronal stem cells, which can migrate into damaged or injured regions. However, the amount of migrating neuronal stem cells is not sufficient for complete regeneration. The development of these neuronal stem cells has initiated the possibility to use them for grafting in brain tissue as a therapy for neurodegenerative diseases [141, 142]. This approach is called stem cell therapy. In the conducted research, the general procedures for grafting and integration of neuronal stem cells into brain tissue have been established. However, the conventional monitoring techniques, microscopy and electrophysiology, can only show that the stem cells become a part of the brain tissue responding to stimulation indicated by recordings from host neurons in the tissue adjacent to the grafted stem cells. These techniques cannot show responses from the entire neuronal network, a part of which the integrated stem cells should become[138]. Furthermore, it is not possible to

confirm whether the integrated neuronal stem cells have become fully differentiated dopaminergic neurons capable of releasing dopamine through exocytosis.

7.5. Filling the gap: Neuroscience meets Micro- and Nanotechnology

Although neuroscience comprises numerous areas with active research, more efforts are needed in research on, e.g., neurodegenerative diseases, cell-cell communication, fate of stem cells during differentiation and influence of blood brain barrier on selection of drug candidates for screening. Conventional biological approaches have, however, certain limitations, one of which is that real-time recordings from growing brain tissue upon selective stimulation of integrating stem cells cannot be performed. Micro- and nanotechnology can help overcome this limitation. Microfluidic tissue culturing can facilitate fluorescence microscopic monitoring of the progress of stem cell integration using GFP producing stem cells while simultaneously conducting potential recordings from the entire neuronal network of the brain tissue. This is possible by fabricating transparent polymeric microfluidic systems which are integrated with micro- and nanoelectrode chips [143]. 3D micro- and nanoelectrode arrays have a great potential as sensors. Microelectrodes having height of about 50 μm can be used for potential recordings from tissue by virtue of their ability to reach into the interior of a tissue slice through the dead cell layer at the bottom of the tissue. At the same time, while the goal of the goal to study the behavior of individual differentiating stem cells, nanoelectrodes also enable addressing subcellular regions. Micro- and nanotechnology are now at the reach of neuroscientists to revolutionize research in medical and diagnostic fields. These advances can bridge the gap between the ultimate goals in neuroscience and the possibility to achieve them.

7.6. Review of research in microfluidics in brain tissue culturing

Rambani *et al.*, have developed a microfluidic chamber for brain slice culturing (Figure 7.4) to culture thick slice (700 μm) [144]. The thickness of the cultured slices made it very challenging to provide a homogenous nutrient supply. To achieve a better viability of tissue, forced convection feeding was utilized in the system. The significance in using thick slices is based on the fact that they have more complex neural networks, high range of synaptic circuits and high polar dendrites, which make them useful for studying multisite stimulation and dynamics of complex neural networks [135].

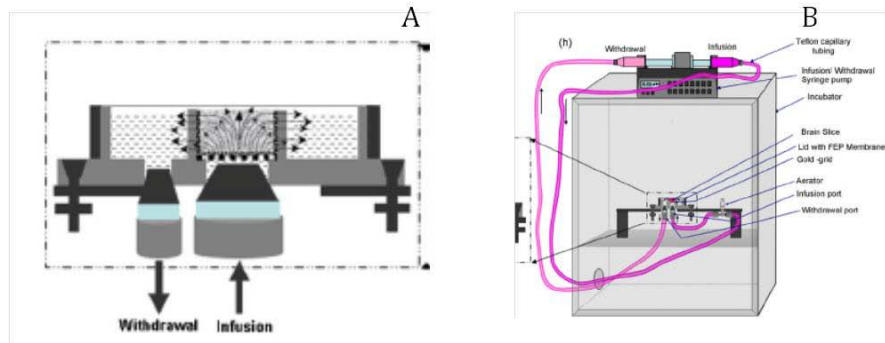


Figure 7.4: A microfluidic brain tissue culture chip with interstitial 3D perfusion.

The image was taken from Rambani *et al.*, 2010 [144].

The microfluidic system comprises an inner culture chamber and an outer waste withdrawal chamber. Culture medium is forced from underneath the chamber through grids containing about 584 openings. The brain slice is placed on the grids. The grid is surrounded by a PDMS well (inner culture

chamber). Channels are fabricated on the well to direct the waste media towards the outer chamber, where it can be withdrawn. An aerator bubble trap is constructed of fluorinated ethylene propylene (FEP) membrane outside the chamber.

The aerator is connected with cartridges that facilitate gas exchange to equilibrate the medium before entering the chamber. The flow rate was optimised for increased tissue viability. The forced convection approach significantly improved tissue viability in comparison with an unperfused slice. The slice culture was successfully maintained for 5 days.

In long-term culturing, thick brain slices (1 mm thickness) always tend to be affected by necrosis due to lack of adequate nutrient and oxygen supply, especially in the mid-section of the slices. This is due to the fact that the capillaries of brain slices used for *in vitro* applications do not provide blood flow as is the case in *in vivo* systems [145]. Based on this, Choi *et al.*, (2007) fabricated hollow microneedles (diameters ranging from 10 to 50 μm and

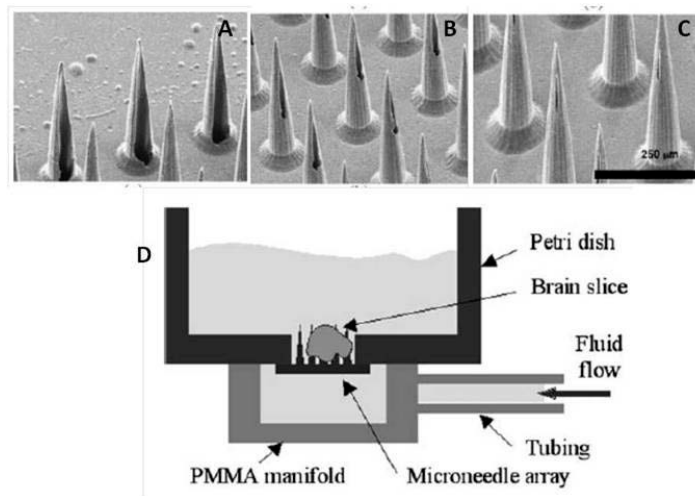


Figure 7.5: Images of microneedles of different diameters. A) 50 μm B) 20 μm C) 10 μm taken by scanning electron microscopy. D) A schematic view of the microfluidic perfusion chamber integrated with hollow microneedles. Modified from Choi *et al.*, 2007 [146].

height about 500 μm) as shown in Figure 7.5A-C.

Although they used 400 μm thick slices, they considered this approach to be necessary to facilitate homogenous perfusion of nutrient medium to the brain slice[146]. The microneedles were fabricated by standard photolithography using SU-8. They were tapered and sharpened by UV light diffraction followed by reactive ion etching. The tip of the microneedles was perforated and connected to channels through the backside of the chip. The microneedle chip was attached to a PMMA microfluidic perfusion chamber to perfuse the nutrient medium (Figure 7.5D). This approach successfully provided the interior regions of the brain slice with an adequate nutrient and oxygen supply. The experiment lasted, however, only for 4 hours. A non-perfused slice was used as a control sample. Dark circular regions were observed in the slice without medium perfusion. Using PI staining, these were revealed to be dead cells.

Blake *et al.*, developed a three layer microfluidic chamber with independent multichannel flow streams on the top and bottom of the layers (Figure 7.6) [147]. The three layers were made of PDMS, which was supported by a glass slide. The PDMS mould was fabricated of SU-8 by standard photolithography. The bottom layer (layer 3) had microposts to provide better adhesion and support of the tissue slice as well as perfusion of medium and gas exchange in order to achieve improved tissue viability. The bottom (layer 3) and top (layer 1) of microfluidic chamber have three inlets. The brain slice is placed in the opening provided by the second layer. For placing the slice in the chamber, the top layer has a flap opening, which can be automatically sealed.

In this work, the main focus was to demonstrate the possibility to examine a localized region of the slice. The effect of chemicals and drugs could be tested in the localized brain region by administrating them through individual inlets. To demonstrate this feature clearly, Na^+ free solution was administrated to deactivate the spontaneous neuronal activity in one half of the medullary

brain slice. At the same time, the other half of the brain region was active. The experiment was conducted only for 4 hours. The paper did not mention how tightly the chamber could be sealed after closing the flap. Improper sealing may cause leakage or bubble formation which could hinder long-term culturing in this type of system.

In 2010, the same group showed the progress of the above described method. They developed a microfluidic chamber, shown in Figure 7.7, which had openings for penetration of commercial multisite electrodes having linear electrode arrays for recording extracellular potentials [148]. The system was fabricated in a similar way as the earlier version utilizing PDMS casted on a

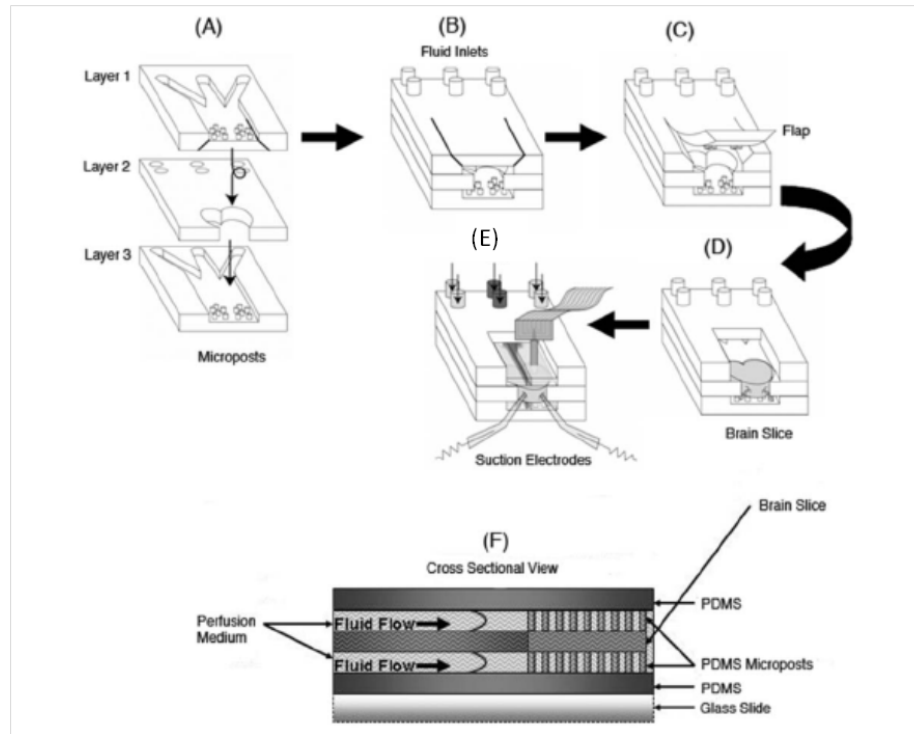


Figure 7.6: Schematic representation of three-layer microfluidic chamber.

A) Three distinct layers. B&C) Assembled three layer with cut a flap on the top layer to place slice. D) Brain slice placed in the chip. E) Insertion of a suction electrodes F) Cross section of. Image taken from [147].

SU-8 mould. It comprised three layers: a bottom with microposts, a middle layer for placing the brain slice and having a lateral opening to insert an electrode to record from the edges of the slice, and a top layer with an opening to insert an electrode in the center of the slice. The system was used for multi-region recordings from the hippocampal region. Numerous reports have been published regarding microelectrode arrays (MEAs), which enable multiple recordings of neuronal activity at microscale [149, 150]. Signals recorded from the interior of neuronal networks are compromised by damage of the tissue during cutting, resulting in the presence of a layer of dead cells on the surface, which directly affects signal-to-noise ratio when using planar electrodes [148, 151]. This can be avoided by three dimensional electrode arrays [134].

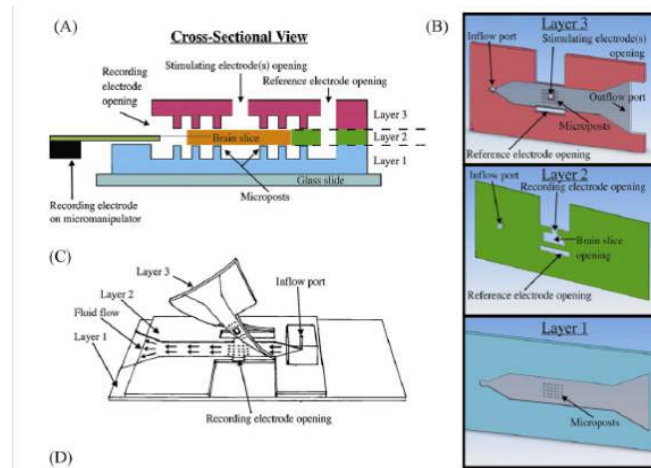


Figure 7.7: Design of microfluidic perfusion chamber

A) Cross section of chamber B) three distinct layer C) assembled three layers (Blake et al., 2010) [148].

In conclusion, the above described applications have addressed individual features of microfluidic tissue culturing, including better tissue survival, forced convection medium delivery to provide improved nutrient and oxygen

supply, integration of multisite electrodes for electrophysiological measurements, and access to localized brain regions. In these described applications, the culture periods ranged from a few hours to 5 days. To obtain *in vitro* cultures that more realistically mimic *in vivo* systems with matured synapticity, it is important to culture brain slices for more than 3 weeks. During the brain tissue preparation, some axonal connections are inevitably cut. This traumatic condition is stabilized with new axonal connections in the course of cultivation [135].

On the other hand, when using acute tissue slices, the locally destroyed neuroanatomy cannot recover from the traumatic conditions. The described systems were used for short-term applications without any evaluation of their applicability and tissue survival during a longer culture period, which would be necessary in applications using tissue slices from p3-p9 animals in combination with integration of neuronal stem cells in the tissue.

8. Long-term Brain Slice Culturing in a Microfluidic Platform⁴

8.1. Introduction

As discussed in section 7.2, the conventional brain tissue culture techniques are used intensively in neuroscience. Organotypic brain tissue cultures employ slices cut from different regions of brain, particularly hippocampus, cortex, cerebellum, spinal cord, and striatum [135]. ometimes co-cultured slices are also used to study the responses between the brain regions [153]. Examples of these are cortico-striatal and cortico-spinal cultures. Slices from animals at the early postnatal stage (p3-p9) are suitable for long-term culturing. The reason is the higher level of neuronal plasticity that gives an improved resistance to mechanical trauma caused by slice preparation using a microtome. In the course of long-term culturing, a complex neuronal network is established. Early postnatal stages (p0-p1) can be also used for *in vitro* culturing but the slices are very tender and lose the morphological features, due to which they can be only used for short-term culturing.

Some of the material has been adopted from our previous pulished data. Vedarethinam Indumathi., Avaliani Natalia., Tønnesen Jan., Hansen J., Sabourin David., Dimaki Maria., Kokaia Merab., Dufva Martin., Svendsen W.E., Emnéus Jenny., Heiskanen Aro., Long-term brain slice culturing in a microfluidic platform. 15th International Conference on Miniaturized Systems for Chemistry and Life Sciences (μTAS 2011), Seattle, Washington, USA, Eds. J.P. Landers, A. Herr, D. Juncker, N. Pamme, J. Bienvenue, Vol. 1, 1560-1563 (2011)[152].

These tissue cultures are often impaired with regards to the supply of nutrients and gas to the inner layers of brain region. Moreover, it is not possible for long term continuous potential recording while cultivating brain tissue. To overcome the issues related with conventional culture techniques, one of the proposed methods is utilization of microfluidic technology to perform brain slice culturing. In recent years, microfluidic systems have been

developed for different applications of brain slice culturing [140, 142-144]. Blake *et al.*, demonstrated a multi layer perfusion chamber which provides nutrients in a controlled manner and targets specific region of brain slice with cellular effectors[147]. Choi *et al.*, developed hollow micro needles, which could possibly be penetrated into the tissue supplying nutrients for the whole of brain slice[135]. Rambani *et al.*, developed a microfluidic system to facilitate culturing a thick brain slice[144]. They cultured thick brain slice (700 μ m) with a forced convection –flow based interstitial perfusion method. Though all these above mentioned methods are interesting in various aspects, they fail to demonstrate long-term culturing.

Long term culturing is essential because the brain slices obtained for *In vitro* cultures are from young animals. Over time from this early age, the basic synaptic connections are progressively matured and form a complex network. For stem cell therapy, the tissue has to be cultured over a long time. In our experiments, It is necessary to culture for longer time, as the neuronal stem cells required 2-3 weeks to start integrating into the brain slice. To address the requirements posed by research toward stem cell therapy of Parkinson’s disease, we have developed a microfluidic tissue culture system to facilitate both long-term tissue culturing and microscopic monitoring of stem cell integration into brain tissue. Ultimately, the system can be integrated with 3D microelectrode arrays for potential recordings from the tissue upon selective stimulation of integrated stem cells.

8.2. Design of the microfluidic systems

Initially a microfluidic system was developed for brain tissue culture. With This knowledge gained, we generated a new generation chip incorporating new features.

8.2.1. Challenges in tissue culturing

For successful implementation of microfluidic technology, the three main challenges that have needed to be addressed are i) easy loading of tissue slices into the culture chambers, ii) maintenance of brain slice culture under proper conditions (sufficient nutrient and O₂ supply as well as pH and temperature control) for at least three weeks, and iii) sufficient optical quality to enable microscopic monitoring of integrating neuronal stem cells.

Formation of air bubbles is a challenging issue in microfluidic systems. Bubbles can be harmful to cells and tissue as well as carry a risk of rupturing the cell membrane [154, 155]. Sometimes bubbles cause drying, which hinders the flow of nutrient medium in that region. Hence, a bubble free culture chip is important for the survival of cells and tissue. In a cell culture system, which has closed culture chambers allowing a cell suspension to be passed through a microfluidic channel, the bubbles are generated during the operation. In the case of a tissue culture system, a chamber needs to be initially open allowing the placement of the tissue slice. When placing the tissue slice, the chamber is filled only partially with the culture medium. Due to this, closing of the chamber leaves a plug of air inside. Hence, an effective air removal system is essential to make tissue loading easy to handle. That was one of the main focuses in designing the microfluidic culture system and is discussed in the following section.

The temperature and gas composition is extremely important for tissue culture system. Mostly the tissue cultures are maintained at 37°C and the gas composition is 95% CO₂ and 5% O₂. To properly maintaining these conditions, is a significant task in order to minimize the onset of necrosis in the inner region of the slice. Therefore, it is essential to use a gas impermeable material for fabricating the culture system. Furthermore, the used fluidic connections and syringes have to be able to maintain the pre-equilibrated gas composition independent of an incubator for a sufficiently long period.

8.2.2. 1st Generation culture system

The 1st generation culture system is a single-chamber system, which was designed to be fluidically operated using a syringe pump and placed on the stage of an upright microscope during tissue culturing (Figure 8.1A). The system is a modification of a previously published microfluidic cell culture system [156]. Figure 8.1B shows a magnification of the culture system, fabricated of 3 layers of 1.5-mm PMMA that were machined using laser ablation and bonded together using UV-assisted bonding under applied pressure and elevated temperature (1 min UV exposure, 88°C, ca. 35 bar for 30 min). It has a dedicated inlet and outlet for medium perfusion and waste removal as well as an opening in the middle to place a brain slice using a large-bore pipette. The opening can be sealed tightly with a 1.5-mm PMMA lid using a laser-cut silicon adhesive gasket. Before sealing the lid, the chamber cannot be filled completely with medium, which results in occlusion of air in the chamber. The retained air can be removed using a funnel shaped structure located in the middle of the chamber (Fig. 45B upper inset), which has a dedicated channel and outlet. The inlet and outlets, made of truncated blunt-end 18 gauge stainless steel needles, are attached in each fabricated system during bonding of the PMMA layers. After bonding the needles are anchored to the complete PMMA structure of the system using two-component epoxy glue to obtain a fluid-tight sealing [156, 157]. To facilitate

an equal lateral flow throughout the chamber, the system has a flow equalizer on both sides of the chamber (Figure 45B lower inset). The chamber height was optimized to avoid shear stress on the tissue. Finite element simulations with COMSOL Multiphysics program (v. 3.4) illustrate the function of the flow equalizer and the optimized height [156].

To facilitate a proper temperature for tissue viability, a Peltier heater (Figure 45A) adapted to the stage of an upright microscope was designed comprising 4 Peltier elements connected in series and sandwiched between two aluminum plates. In order to get the feedback for temperature control from a chamber having perfusion of culture medium, a thermistor was inbuilt inside the chamber of a dedicated system that was placed on the Peltier heater beside the system used for tissue culturing. This was necessary to avoid the influence of the thermistor on the tissue in the chamber used for culturing.

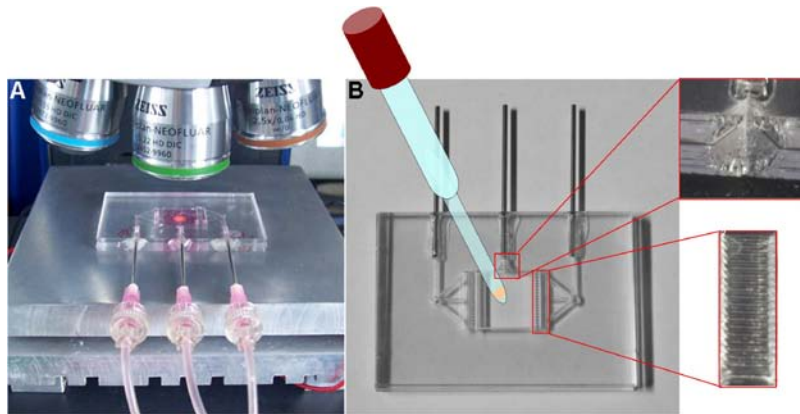


Figure 8.1: 1st generation of brain tissue culture system

(A) A single-chamber brain slice culture system designed for culturing on a microscope stage having a Peltier heater. (B) A schematic view of tissue loading into the culture chamber and magnification of the chamber design with a funnel shaped channel (upper inset) and flow equalizers (lower inset).

8.2.3. 2nd generation culture system

The 2nd generation tissue culture system was based on a new design with modified features to improve the operational and functional properties of the 1st generation culture system. The necessary modifications were designed based on the experimental findings during culturing in the 1st generation system. Figure 8.2 shows the structure of the new design, which comprises

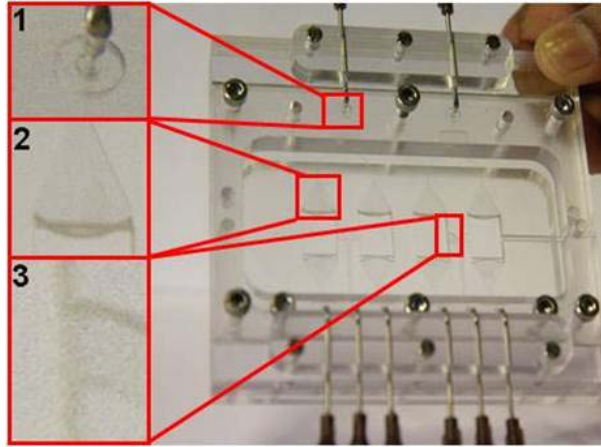


Figure 8.2: A multi-chamber brain slice culture system comprising a fluidic connection board and culture chip interfaced to each other using PDMS O-rings (inset 1). Magnification of a flow equalizer (inset 2) and a funnel-shaped channel (inset 3).

two main features, i) a reusable fluidic platform and ii) a disposable tissue culture chip. This system was designed for culture experiments on the stage of an upright microscope facilitating temperature control using the Peltier heater designed for the 1st generation culture system (Figure 8.1A).

To simplify the assembling process during preparation of a culture experiment, a “plug-and-play” approach was developed, which facilitates easy placement of a new culture chip on the fluidic platform having the necessary

fluidic connections. After plugging in a culture chip, the platform has a screw-tightened frame to lock the chip to ensure uninterrupted operation during experiments. The inlets and outlets for connecting the system to a syringe pump were made of 20 gauge stainless steel needles anchored directly in precisely dimensioned apertures on the upper side of the fluidic platform. The dimensions of the apertures facilitated liquid- and gas-tight needle attachment without using any glue. To ensure free access to the surface of the system during microscopic monitoring, the needles were bent.

The used tissue culture chips were fabricated using 3 layers of 1-mm PMMA. The disposable chips have 4 chambers, which allow performing 3 simultaneous culture experiments. One of the chambers has a dedicated side channel for insertion of a thermistor for temperature feedback during experiments. Due to the presence of the thermistor, this chamber is not loaded with tissue. It is fed, nevertheless, with medium to ensure temperature control under precisely the same condition as the one in the chambers loaded with tissue. In order to improve microscopic monitoring, the optical path through the chip was shortened. This was achieved by using 1-mm PMMA instead of 1.5-mm PMMA as was the case for the 1st generation system. Furthermore, the windows used to close the chambers after tissue loading were made of 0.5-mm PMMA. Placement of the tissue and sealing of the PMMA window was designed according to the same procedure as in the case of the 1st generation system.

When attaching the culture chip on the fluidic platform, the tightness of the fluidic connection is ensured using tailor-made O-rings (Figure 8.2, inset 1). The PDMS O-rings are imbedded in the upper surface of the fluidic platform and the lower surface of a culture chip to ensure that they are not exposed to the ambient. Hence, the gas permeability of PDMS does not compromise the gas-tightness of the assembled system. The flow equalizer was modified to simplify the fabrication. Figure 8.2 (inset 2) shows a magnification of the

structure, which comprises an expansion chamber with the same depth as the fluidic channels (250 μm) and a curved edge that forms a step-like portion down to the bottom of the culture chamber. The initially retained air, when closing the chambers using the PMMA window, is also removed through a funnel shaped structure (Figure 8.2, inset 3), the shape and dimension of which was slightly modified from the ones used in the 1st generation system.

The features described above, i.e. the functional plug-and-paly approach and fluidic sealing using individual O-rings for each culture chamber, require very precise fabrication to achieve a sufficient accuracy for alignment. Due to this, micromilling was used for machining of all the components of the entire system. Micromilling also helped in precise control over the geometry and dimensions of the fluidic channels of the culture chip (width: 500 μm ; depth: 250 μm) as well as positioning of the apertures of the fluidic platform.

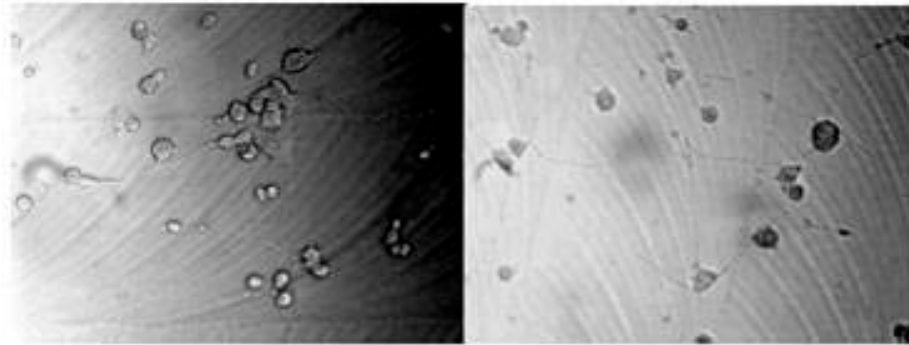


Figure 8.3: Microscopic images of differentiating PC12 cells
5 h (left) and 24 h (right) after cell seeding. The differentiation was conducted
on the stage of an upright microscope.

Since there is no animal facility available at DTU, the function of the developed system in general and the reliability of the designed features were initially tested using cell culturing. Cell culturing in microfluidic systems is a

significant part of the EXCELL project, providing the necessary expertise and experience, thus facilitating a routine operation to function as an effective assessment method. Rat pheochromocytoma (PC12) cells were chosen as the model cell line for the test using medium containing nerve growth factor (NGF), to which PC12 cells respond by outgrowth of neurite-like structures (differentiation) [158]. This test addressed the function of the culture system design in terms of i) temperature control using the Peltier heater previously developed for the 1st generation culture system, ii) gas impermeability to retain the desired gas composition of the culture medium during operation, iii) the flow conditions in the culture chambers, and iv) the optical clarity to visualize individual cells. The progress of PC12 cell differentiation in terms of morphological changes and outgrowth of the neurite-like structures was monitored using time-lapse microscopy. The test demonstrated that the system could be operated on a microscope stage independent of a CO₂ incubator to monitor continuously the cells and that the flow rate used with the 1st generation system (75 μ l/h) was suitable. Figure 8.3 shows images taken 5 h (left panel) and 24 h (right panel) after cell seeding. The cells were responding to NGF with observed neurite-like outgrowths 24 h after cell seeding.

8.2.4. 3rd generation culture system

Traditionally, microfluidic cell/tissue culture experiments are performed using a pressure driven pump, such as a syringe pump. Though the microfluidic devices are small, the pumps are significantly larger. When increasing the number of culture chambers in a microfluidic system, several pumps may be required to perform an experiment unless a multi-channel syringe pump is available. Under these conditions, even though the microfluidic systems can be easily placed and operated on a microscope stage, the placement of syringe pump(s) beside the microscope is a complicating factor. In the case of the 2nd generation culture system, when culturing was

performed using several systems parallel, it was not possible to place them on the microscope stage with connections to a sufficient number of syringe pumps. Such an experiment had to be performed by placing the systems in an incubator and the syringe pumps in front of the incubator (Figure 8.4). Even in this case, the arrangement was difficult to handle.

Another limitation imposed by the usage of syringe pumps is the fact that syringes have a dead volume in terms of the operation of a pump. This means that it is not possible to use the entire volume placed in a syringe, which can also increase experimental expenses if expensive reagents are needed. Furthermore, the usage of syringe pumps also complicates the operations when the culture medium needs to be changed.

In order to overcome the operational limitations and complications when using microfluidic systems driven by syringe pumps, it is desirable to have a



Figure 8.4: A tissue culture experiment using several 2nd generation systems operated by syringe pumps (Photography courtesy of Dr. David Sabourin).

system comprising multi-channel micropumps on the same platform, which also houses the necessary fluidic connections and the culture chip. A versatile multi-channel micropump adaptable to different microfluidic platforms has been developed in-house [10]. Such pumps have also been integrated on a microfluidic platform used in the EXCELL project for cell culturing and differentiation (Figure 8.5A). The platform houses, aside from the micropumps, also the motors running them as well as liquid reservoirs for culture medium and waste collection, which enable easy liquid handling. A

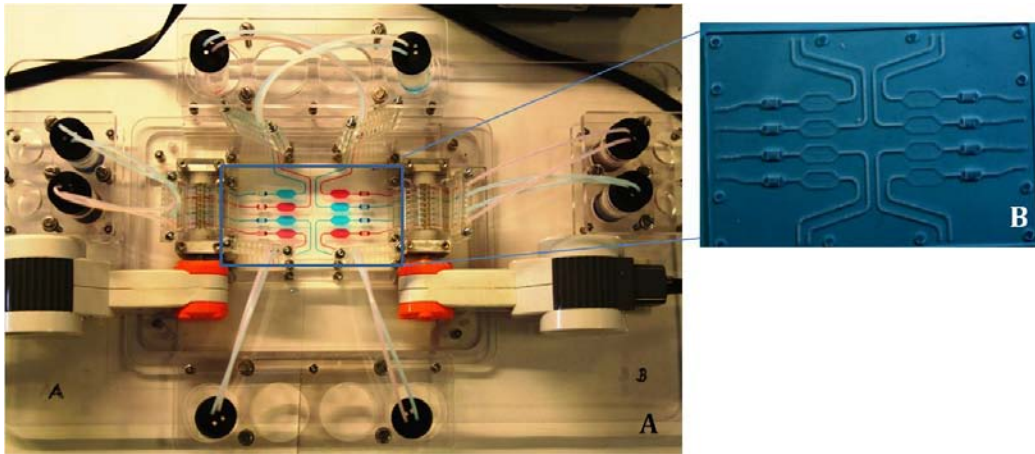


Figure 8.5: A micro pump system integrated with a cell culture chip.

A)Micro pump system (B) An array cell culture system.

tailor-made culture chip (an enlarged image:Figure 8.5 B) can be placed in the middle of the platform. Since this platform has PDMS ribbons in the pumps to enable the peristaltic pumping, the system is not, at present, suitable for operations on a microscope stage unless this is equipped with an incubator function. Nevertheless, adaptation of this platform to tissue culturing was considered appropriate in order to overcome complications using syringe pumps. Since the platform is also highly portable, it is easy to

take it to a microscope for imaging and to a laminar flow bench for changing the medium.

The culture chip for the 3rd generation culture system was designed and machined in the same way as the 2nd generation culture chip with adaptation to the platform having micropumps (A and B). In Figure 8.5, the different components of the microfluidic platform are indicated by yellow frames. The inlet tubing from medium reservoirs (1) is connected to PDMS ribbons that are placed inside the micropumps (2), operated using step motors (3) from LEGO MINDSTORMS, Denmark. The inlet tubing is inserted inside vials, which are tightly closed with a cap having a rubber septum. To avoid creation of vacuum during liquid pumping, 400- μ m needles with filters are attached to the septa to allow gas equilibration and pressure adjustment. Using this approach, it is easy to maintain the culture medium sterile during operation. Furthermore, it is easier to change the culture medium in comparison with syringes attached to a syringe pump. The other end of each PDMS ribbon is connected to the microfluidic tissue culture chip in the center of the platform. Another set of PDMS ribbons (4) connects the microfluidic chips to the outlet tubing leading to the waste reservoir vials (5). During microscopic imaging, the entire system can be taken easily to the microscope stage without stopping perfusion. Flow rates during medium perfusion can be adjusted using a LabView-based software from Lego. Figure 8.5B shows a magnified view of the culture chip, which comprises (1) bubble traps to eliminate the entrance of bubbles generated during medium perfusion, (2) flow equalizers having the same structure as in the microfluidic chips for the 2nd generation system, and (3) funnel shaped structure for removal of the initially retained air. As in the case of the 2nd generation system, the chambers are closed using 500- μ m PMMA windows attached using a silicon adhesive gasket.

As in the case of the 2nd generation system, differentiation of PC12 cells was performed in the system to assess its functional properties. PC12 cells were differentiated in all the chambers of the system. During the first 24 h after cell seeding, the cells were perfused with culture medium. Figure 8.7 shows the cells 90 min (A) and 21 h (B) after seeding. The characteristic initially

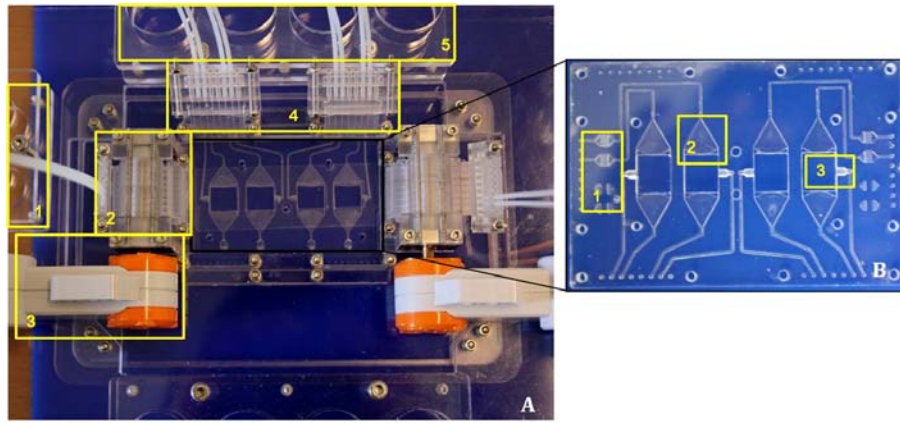


Figure 8.6 A) Microfluidic platform adapted for tissue culturing. The system comprises vial holders and vials for medium perfusion (1) and waste collection (5), micropumps with PDMS ribbons (2), step motors for pump operation (3), PDMS ribbons (4) for connecting the outlet tubing. B) A magnified view of the 3rd generation microfluidic brain slice culture chip having (1) bubble traps, (2) flow equalizers and (3) funnel shaped structures to remove the retained air.

round shape of PC12 cells was changed to the normal morphology of adherent and proliferating cells imaged after 21 h. 24 h after seeding, the medium was changed to differentiation medium containing NGF and perfusion was continued for 5 days. Figure 8.7 C-E show the progress of differentiation in response to NGF imaged 48 h, 72 h and 144 h after cell seeding, respectively. The result of this test indicated that the developed 3rd generation system

could be suitable for brain tissue culturing facilitating an easier operation during the experiments.

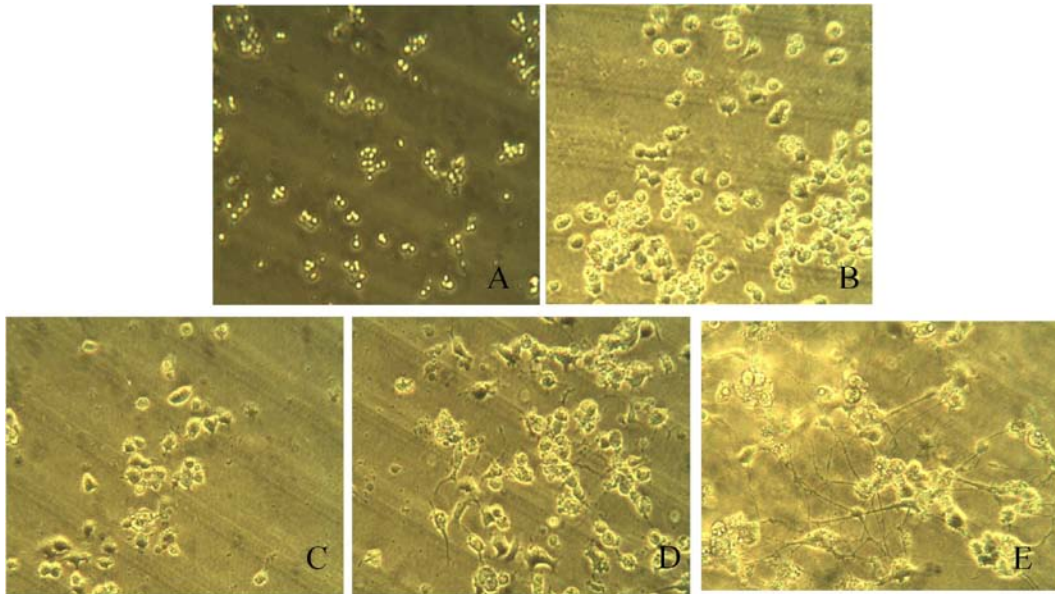


Figure 8.7: Microscopic images of growing and differentiating PC12 cells in a microfluidic system A) 90 min, B) 21 h, C) 48 h, D) 72 h and E) 144 h after cell seeding. Cells shown in images C) - E) have been perfused with differentiation medium for 24 h, 48 h and 120 h, respectively.

8.3. Window material for tissue culture chips

After placing a tissue slice in each of the chambers of a culture chip, these have to be closed using a fluid-tight lid to allow medium perfusion. The closing process needs to be handled easily and fast to avoid any delay and mechanical stress that can affect tissue viability. Furthermore, the lid has to

possess a sufficient optical quality to function as a window during microscopic imaging. Three different materials, glass cover slips (thickness: 200 μm), PMMA (thickness: 500 μm) and Pyrex glass (thickness: 500 μm), were assessed in terms of the closing process and the optical quality of the material.

Glass cover slips are very fragile; hence, no pressure can be exerted during the closing process. The only suitable means to seal cover slips gently was gluing with histoacrylic tissue adhesive. The obtained sealing was fluid-tight and the optical quality for tissue visualization was very good. However, application of cover slips was complicated by their fragility during culture experiments as well as the low viscosity of the glue, causing leakage into the culture

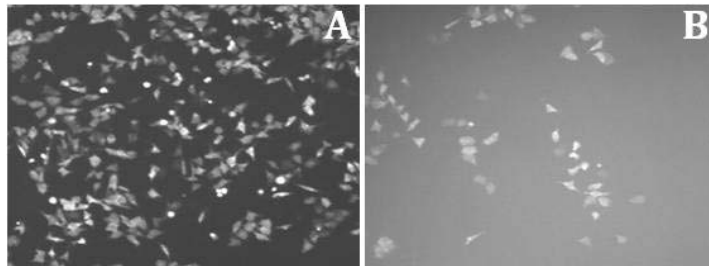


Figure 8.8: Testing PMMA substrate as sealing window.

A) HeLa cells with PI staining on the culture chip without media B) HeLa cells with PI staining on the culture chip sealed with PMMA lid and filled with media.

chambers. Microscopic images of HeLa cells through PMMA and Pyrex lid, respectively, in chambers filled with culture medium. Based on the chosen criteria, the closing process and optical properties, it was not possible to determine which material would be more preferable for tissue culture experiments. However, considering the significantly higher price of Pyrex

material as well as the higher instrumental cost when cutting tailor-made Pyrex windows for the culture systems, PMMA was used in experiments conducted with both 2nd and 3rd generation tissue culture systems.

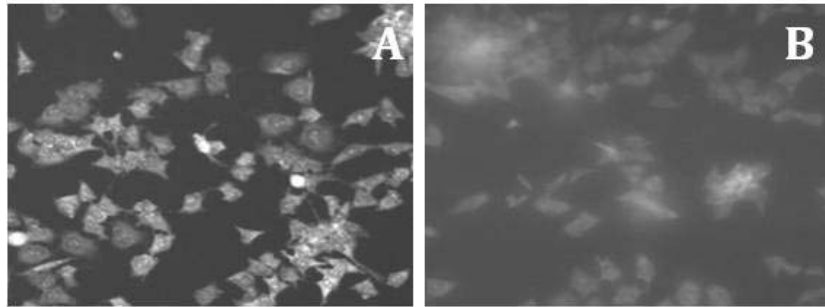


Figure 8.9: Testing Pyrex glass substrate as sealing window
A) HeLa cells with PI staining on the culture chip without media B) HeLa cells with PI staining on the chip sealed with Pyrex glass lid and filled with media.

8.4. Experimental

8.4.1. Preparation of culture systems

The microfluidic systems were sterilized using 0.5 M NaOH for 15 min followed by thorough rinsing with cell culture tested water and PBS. Each culture chamber was coated with poly-D-lysine (10 $\mu\text{g}/\text{ml}$) for 2hrs at room temperature and laminin (20 $\mu\text{g}/\text{ml}$) overnight at 37°C in an incubator.

8.4.2. Brain slice preparation

Brain slices were derived from two kinds of animal models: i) Balb/c mice expressing GFP coupled to the expression of the gene encoding for glutamate decarboxylase (GAD) (referred to as GAD-GFP mice) and ii) mice without GFP production (referred to as null mice). GAD-GFP mice were used for experiments testing only tissue culturing to enable visualization of the tissue and assessment of its viability, whereas null mice were used for experiments testing integration of neuronal stem cells that were genetically modified to produce GFP to enable their visualization in the brain slice.

A vibratome was used to prepare 250 μm thick brain slices from p8 mice. The preparation of tissue slices was done based on standard procedures according to the guidelines followed at Lund University (Stockholms Norra Djurforsöksetiska Nämnd, Stockholm, Sweden; N150/05 and N154/06). In order to start a microfluidic tissue culture experiment, one tissue slice was transferred into each of the utilized culture chambers using a large-bore glass pipette (schematically visualized in Fig. 8.1B).

8.4.3. Removal of retained air

After placing the tissue slices in the chambers, each chamber was half-filled with culture medium. Prior to closing the chambers, the culture systems were placed in a CO_2 incubator for 15-30 min to allow adhesion of the slices on the coated surface. The chambers were then closed with a PMMA window using a double-sided adhesive silicon gasket to ensure fluid-tightness. The air retained in the chambers upon closing the window was removed through the dedicated channels having a funnel shaped opening (Fig. 8.1, 8.2 and 8.6) by slowly perfusing culture medium. During medium perfusion, the channel leading to this structure was closed with a 3-way valve.

8.4.4. Perfusion culture operation

Prior to filling a syringe with culture medium, this was first equilibrated in an incubator to obtain a proper oxygen and CO₂ composition. This procedure was necessary when using the gas impermeable 1st and 2nd generation culture systems. The used syringes were equipped with a Luer lock to interface them easily to tubing with 3-way valve. Medium to each culture chamber was delivered from a dedicated syringe operated by a laboratory syringe pump. Different flow rates were tested during the experiments. The culture medium in the syringes was changed every second day to ensure that the desired gas composition was maintained. Temperature during culturing on microscope stage (1st and 2nd generation culture systems) was maintained using a custom-made Peltier heater controlled by a TC-36-25 temperature controller (TE Technology, MI, USA). In experiments using several parallel 2nd generation systems and 3rd generation system having gas permeable PDMS components, a CO₂ incubator was used for temperature control.

8.4.5. Microscopic imaging

Microscopic imaging during tissue culturing was done using either an inverted Olympus microscope or an upright Zeiss microscope.

8.5. Result and Discussion

8.5.1. Brain slice culturing in the 1st generation system

The aim of the conducted research has been to develop a microfluidic culture system enabling tissue culturing for a sufficiently long time, at least three weeks, to facilitate integration of neuronal stem cells in brain tissue and their

differentiation to dopaminergic neurons. In order to conduct monitoring using time-lapse microscopy, the culture system has to be operated independent of a CO₂ incubator. Hence, the system needed to be capable of maintaining the optimal temperature and gas composition. The chosen material for fabricating the system was gas impermeable PMMA.

Perfusion culture of a hemispherical brain slice on the microscope stage in the culture system depicted in Figure 8.10 was continued for 6 days. Since temperature control during culturing was done using a Peltier heater, which is not transparent, the culture system was placed on the stage of an upright microscope. The culture chamber of the fabricated system comprises 2 pieces

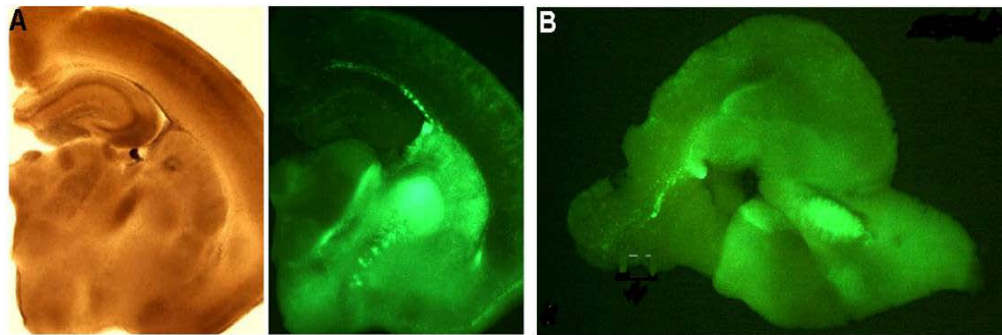


Figure 8.10: Microscopic images of a hemispherical brain slice from a P8 transgenic GAD-GFP mouse cultured in the system shown in figure Figure 8.10 for A) 20 h (left panel: bright field; right panel: GFP fluorescence) and B) 6 days (GFP fluorescence). Imaging was done using an inverted Olympus microscope.

of 1.5-mm PMMA plates. The lid closing the culture chamber is also 1.5 mm thick PMMA. Hence, microscopic visualization of the slice needs to be done through a liquid layer of nearly 3 mm and a PMMA layer of 1.5 mm. Due to this distance, real-time microscopic observations could not be done properly. In order to acquire microscopic images of the progress of the culturing, the

system had to be taken to an inverted microscope, which could facilitate a sufficient optical clarity through the 1.5 mm thick PMMA bottom of the system. Figure 8.10 shows microscopic images of the cultured tissue slice 20 h (Figure 8.10A) and 6 days (Figure 8.10B) after starting the perfusion.

Although the system could successfully sustain tissue culturing for 6 days, the obtained results indicated a clear decrease in GFP fluorescence during the culture period. In order to extend the culture period beyond 6 days, a major concern was the possibility that the epoxy glue, which was used to anchor the needles for inlet and outlet channels, could get into contact with the culture medium. Despite the fact that a similar approach had been successfully used in fabricating a cell culture system and the conducted experiments [157, 159], cell culture tests in several reproduced 1st generation tissue culture systems (results not shown) indicated the manifestation of toxic effects that were attributed to the influence of the epoxy glue.

Despite the problems in visualization of the tissue using an upright microscope and the possibility of toxic effects due to contact of culture medium with the epoxy glue, the system functioned well during the operation of perfusion culture. When the tissue had been placed in the culture chamber and the lid had been sealed using the silicon adhesive gasket, the retained air could be readily removed through the funnel shaped channel shown in the upper inset of Figure 8.1B. The culture chamber remained bubble-free throughout the culture period even when medium was changed. Based on previously published finite element simulations and conducted flow tests in a system similar to the 1st generation tissue culture system [156], the chosen flow rate of 75 $\mu\text{l/h}$ was considered to be safe for the cultured tissue.

However, to facilitate time-lapse microscopic monitoring of tissue cultures using an upright microscope, which is compulsory when using a nontransparent Peltier heater, and to ensure that no glue was needed for

anchoring the inlet and outlet connections, further development of the 1st generation culture system was necessary.

8.5.2. Brain slice culturing in the 2nd generation system

In order to improve the optical properties of the culture system, it was necessary to decrease the thickness of the liquid layer and the PMMA lid/optical window, through which the microscopic visualization using an upright microscope had to be done. 1 mm thick PMMA was chosen for the fabrication and 500 μm thick PMMA for the optical window. In the case of the 1st generation culture system, the thicker PMMA was chosen to facilitate the anchoring of the needles serving as inlet and outlet connections. To be able to use thinner PMMA, usage of needles in each individual culture system had to be eliminated. At the same time, this could also help in eliminating the necessity of gluing. To achieve this, a reusable fluidic connection board (Figure 8.2) was designed to be used with disposable culture chips.

The application of the fluidic connection board required a higher precision for the fabrication of the culture chips to allow accurate alignment of the inlet and outlet channels to the corresponding positions on the fluidic connection board. Due to this reason, laser ablation, which lacks the sufficient precision in X-Y control, could not be used. The desired precision could be achieved using micromilling. Aside from the improved precision when using micromilling, this was also the only technique that could facilitate the fabrication of the fluidic connection board that had to be made of a sufficiently thick polymer (at least 8 mm).

Since the conducted tests using cell differentiation (described in section 8.2.2) indicated that the 2nd generation system had a reliable fluidic function, optical clarity and temperature control using the Peltier heater, it was applied for tissue culturing. In the first tissue culture experiment, hippocampal tissue slices were cultured for 16 days. Figure 8.11 shows microscopic images after the 16-day culture period using an upright microscope. Despite the fact that the system showed improved optical quality due to shorter optical path and optimal microfluidic function for handling of tissue culture, onset of local

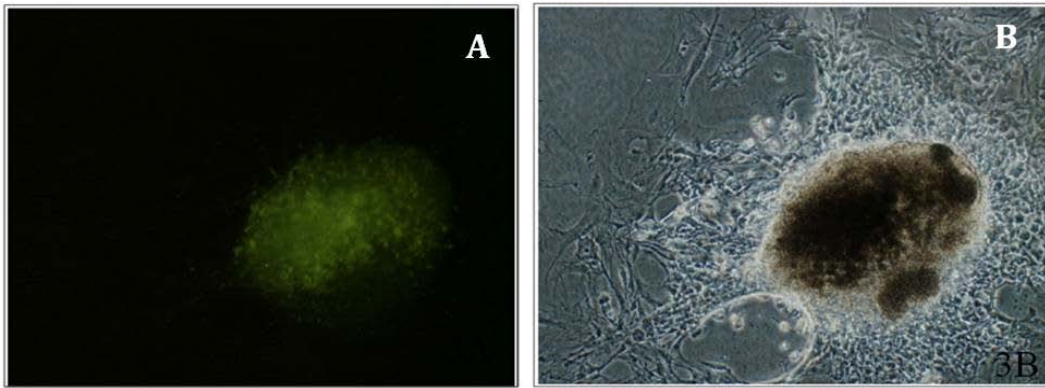


Figure 8.11: Microscopic images of a hippocampal tissue slice from a P8 transgenic GAD-GFP mouse cultured for 16 days using the culture system shown in figure 46. Necrosis was observed (dark regions in B)) in the tissue. A) Fluorescence and B) bright-field images, were done using upright Zeiss microscope.

necrosis was observed. This could be visualized, however, only by using bright-field microscopic imaging (Figure 8.11B) although fluorescence microscopy of GFP after 16 days in culture (Figure 8.11A) did not indicate any sign of necrosis.

Since bright-field imaging was done only in the end of the experiment, it is not possible to determine at what point during the culture period the onset of necrosis occurred. However, considering the observed progress of necrosis, seen in Figure 8.11B, it must have occurred a few days after the start of the experiment. Necrosis is cell death due to lack of oxygen supply, caused by external factors, such as infections, toxins and trauma[135]. Since tissue culturing in a microfluidic system, including both preparation and operation

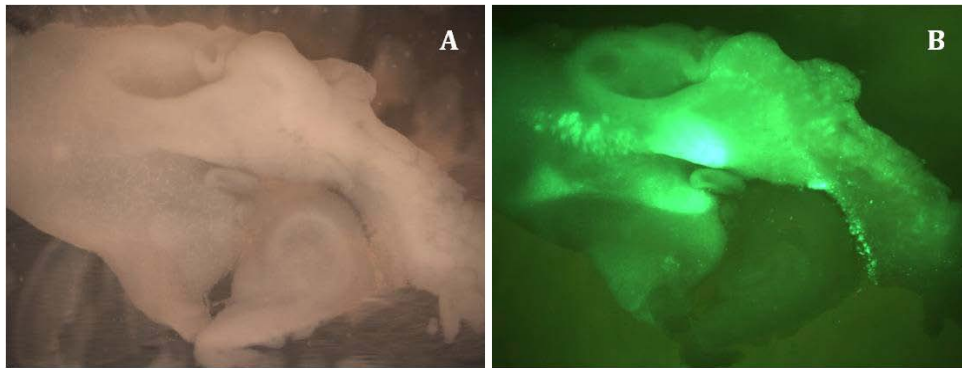


Figure 8.12: Microscopic images of a hemispherical brain slice from a P8 transgenic GAD-GFP mouse cultured for 8 days in the system shown in figure 8.2. A) Bright-field and B) fluorescence images, were done using an upright Zeiss microscope.

of the culture, is a more complicated process than microfluidic cell culturing, various reasons can have caused the onset of necrosis. The factors that were considered to require improvement were the initial preparation of a tissue culture experiment and the operational conditions.

Preparation of a tissue culture experiment comprises five primary steps: i) tissue slice preparation, ii) placement of a tissue slice in each of the culture chambers, iii) adhesion of the tissue slices, iv) closing of the culture chambers,

and v) removal of the retained air. A delay or technical complication in any of these steps may directly contribute to onset of necrosis. In the conducted experiment using this 2nd generation system, three significant differences in comparison with the experiment previously conducted using the 1st generation system was observed.

For proper microscopic observations, the placed tissue slice has to be as close to the center of the culture chamber. Since tissue slice preparation and placement in the culture chambers cannot be done at DTU, the conducted experiments were dependent on a collaborator at Lund University. After the experiment using the 1st generation culture system, a new person took over the responsibility for tissue preparation and placement in the culture chambers. In this first experiment using the 2nd generation culture system, the placed tissue slices initially floated to the periphery of the chambers. To ensure visibility during microscopic monitoring, the slices had to be moved toward the center of the chambers causing mechanical agitation that can have contributed to the onset of necrosis. To eliminate the possible influence of this factor, separate tests were conducted to improve the necessary skills in placing tissue slices in culture chambers.

Proper adhesion of the tissue slices on the bottom of culture chambers prior to closing the lid is necessary for a successful experiment. Lack of sufficient adhesion can severely influence the subsequent steps in preparation of the experiment as well as the subsequent fluidic operations. Since for this experiment, the available tissue slices were hippocampal slices, which are significantly more compact than hemispherical slices, the reduced surface area of a slice in contact with the bottom of a culture chamber clearly decreased the effective adhesion. Although hippocampal tissue slices as organotypic cultures could give specific biological information, they complicate the experimental procedure in terms of decreased adhesion. Since the conducted research had to first establish an optimally functioning culture system and

microfluidic operations, hemispherical tissue slices were chosen for further experiments. After completed development and optimization, it could be possible to conduct experiments using organotypic cultures.

If the removal of the retained air cannot be done smoothly, the process can cause additional agitation of the tissue slices. In the course of the preparation of this first experiment with the 2nd generation culture system, it was noticed that funnel shaped channel that had been fabricated using micromilling did not function as effectively as the one fabricated using laser ablation in the 1st generation culture system. The major reason for this was due to the fact that both the profile and dimension of the funnel structure, when using micromilling, are dependent on the chosen fabrication tool. Separate tests were conducted to improve these features. The result of this optimization was a bell-shaped structures with diameter nearly the same as the height of the culture chamber. This structure ensured an effective removal of the retained air and was implemented in the culture chips for continued experiments.

With regards to operational conditions during experiments, further optimization of the flow rate was considered as the most significant. The effect of flow rate can be tested only by conducting a complete culture experiment. Hence, testing of the effect of several different flow rates, which is significant in order to achieve the best operational conditions, would be very time consuming and labor intensive. Since the culture chambers of the 2nd generation culture system were shallower than the chamber of the 1st generation culture system, decrease in flow rate from the initially used 75 $\mu\text{l/h}$ to 15 $\mu\text{l/h}$ was chosen to eliminate possible agitation of tissue slices due to too high a flow rate, which could lead to necrosis and detachment of the tissue.

The conducted culture experiment using a 2nd generation culture chip with the optimized features described above at flow rate 15 $\mu\text{l/h}$ was continued for

8 days. Figure 8.12 shows microscopic images of a tissue slice after 8 days in culture. Based on the conclusion described above that the onset of necrosis has to occur already during the early part the culture period, this experiment was terminated after 8 days since no visible signs of necrosis were observed in bright-field microscopic visualization (Figure 8.12A). Furthermore, the intensity of GFP fluorescence remained strong throughout the culture period (Figure 8.12B). Aside from the improved viability of the tissue slices under the operational conditions in this experiment, the other consequence was that no signs of tissue detachment during the culture period were observed. As mentioned above, a complete optimization of flow rate needs to be conducted as part of future experiments. Additionally, electrophysiological measurements need to be conducted on cultured tissue to assess the functional properties of tissue slices after perfusion culture.

The result of this second culture experiment provided important additional information on microfluidic experiments using biological material. During the development of the 2nd generation culture system, the general functionality of the system was tested by differentiating PC12 cells in the chambers (shown in section 8.2.2). Based on that test, including the used flow rate 75 $\mu\text{l/h}$, cell differentiation functioned optimally based on microscopic observations. Despite this test, several factors needed further optimization to achieve proper functionality and conditions for tissue culturing. This indicates that tissue slices are more prone to damage, requiring much more optimized system features and operational conditions than cultured/differentiated cells.

8.5.3. Brain slice culturing in the 3rd generation system

The culture chip structure that was optimized as part of the 2nd generation culture system was modified to adapt it to the micropump platform developed for cell culturing in the EXCELL project. With this format, the resulting 3rd

generation tissue culture system would have a highly compact structure facilitating fluidic delivery through multiple channels eliminating the necessity of placing large syringe pumps around a culture system. Aside from the compact parallelized structure, the system could enable easy microfluidic operations during culture experiments.

Despite a successful cell differentiation experiment using the developed 3rd generation culture system (described in section 8.2.3), the system failed in conducted tissue culture experiments. Upon analysis of the observed problems, two aspects were considered as the main reason for the failure: i) incomplete sealing of the lid and ii) inability to remove effectively the initially retained air in the culture chambers. To seal the lid using the silicon adhesive based on the approach successfully tested using the 1st and 2nd generation culture systems a sufficiently high pressure is needed to form a liquid tight sealing. In the 3rd generation system, the culture chip is not supported underneath to allow application of the necessary pressure. Increased pressure during the sealing process was noticed to affect the fluidic connections to the culture chip causing leakage of medium during perfusion. If the disruption of the fluidic connections could be avoided, the sealing was not functional resulting in access of air into the chambers with the concomitant generation of small bubbles that relatively fast filled the culture chambers. Partially due to the improper sealing of the lid as well as inability of the micropumps to create a sufficient pressure during operation, the initially retained air could not be removed completely. The combined effect of these problems was manifested in decreased tissue viability due to the presence of bubbles in the chambers and incomplete medium perfusion. Furthermore, the bubbles filling the chambers also hindered microscopic observations.

Although the 3rd generation system based on the existing pump platform was not suitable for tissue culture experiments, the expected benefits justify modifications of the platform to eliminate the observed problems. This was

not possible, however, as part of the conducted research and further structural optimization of the 3rd generation system will be conducted in future work.

8.6. Integration of neuronal stem cells

The 2nd generation system with the optimized structure under the successfully tested flow conditions was used for experiments toward integration of stem cells into cultured brain tissue. In order to evaluate first the possibility to visualize neuronal stem cells during integration in cultured brain tissue, a preliminary experiment was conducted using homogenized brain tissue with GFP fluorescence. The tissue fragments were placed on non-fluorescent tissue slices prior to closing the culture chambers. Figure 8.13 shows a fluorescence image of tissue fragments on a tissue slice, indicating the possibility to visualize individual neurons. Since the fragments did not integrate in the tissue, they were flushed away however during continued perfusion.

The experiment to test integration of neuronal stem cells into brain tissue under microfluidic conditions was started by culturing a non-fluorescent brain slices from a P8 mouse for 24 h. After the initial brain slice culture period, GFP producing neuronal stem cells were seeded into the chambers through the channel leading to the funnel shaped structure. Although these channels were designed for optimal removal of the initially retained air, they proved to be well suited for cell seeding. Directly after cell seeding, the system was left without perfusion for 1 h to allow adhesion of the seeded cells in the tissue, after which perfusion was continued at the rate of 15 $\mu\text{l/h}$.

Figure 8.14 shows neuronal stem cells integrating on brain slice after 2 days of perfusion. During continued perfusion, the number of stem cells on the tissue decreased and after 4 days of perfusion only a few cells could be observed. The reason for the decreased cell number during continued perfusion is not

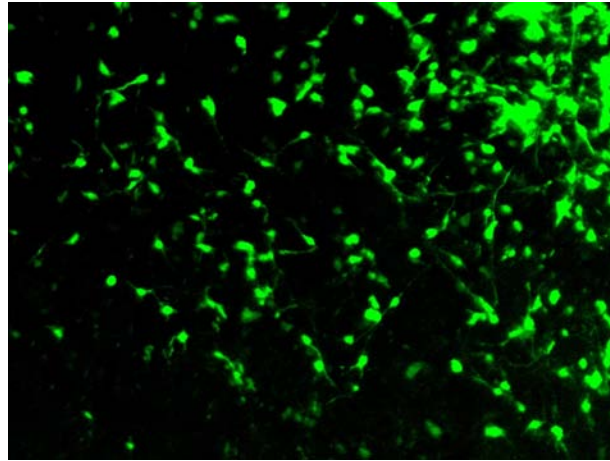


Figure 8.13: A fluorescence microscopic image of homogenized brain tissue from a P8 transgenic GAD-GFP mouse placed on a nonfluorescent brain slice in a culture chamber of the 2nd generation culture system. Imaging was done using an upright Zeiss microscope.

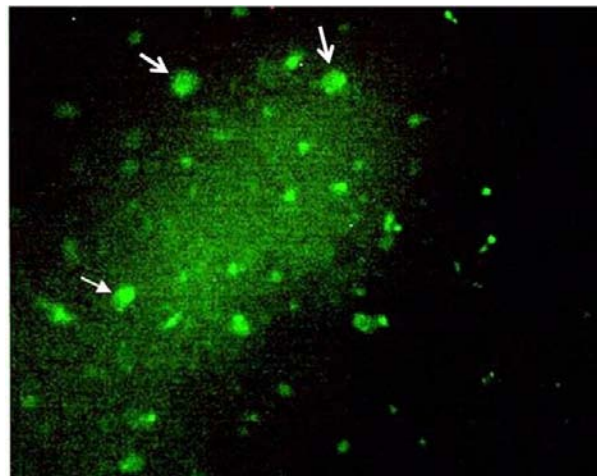


Figure 8.14: A fluorescent image of GFP producing neuronal stem cells integrating into a nonfluorescent brain slice after 2-day perfusion in a culture chamber of the 2nd generation culture system. Imaging was done using an upright Zeiss microscope.

fully clear but it can be attributed to such factors as i) too short an initial period without flow affecting the ability of the cells to adhere properly on the tissue and ii) too high a flow rate during the perfusion.

To incubate the cells for a longer time without perfusion is easily affected by increased acidification, which can counteract the expected outcome. To decrease the flow rate from the used 15 $\mu\text{l/h}$, which functioned with tissue culturing, can also increase acidification since waste materials are not removed as effectively. However, it may be sufficient to use a decreased flow rate during a shorter period when the stem cells still are on the surface of the tissue. This is expected to aid in retaining the factors secreted by the stem cells and needed for their differentiation. Although this initial experiment to integrate stem cells did not function optimally, the obtained result is promising and forms the basis for continued work to optimize the procedure. In order to succeed in both integration of stem cells and their subsequent differentiation during the longer required perfusion period, it is important that a sufficient number of stem cells can be retained on the tissue after cells seeding

8.7. Conclusion

A series of microfluidic systems were developed for long-term brain slice culturing. Using the 1st generation culture system, the possibility to succeed in culturing on a microscope stage with optimal culture conditions was demonstrated as the prerequisite for real-time imaging. The system required, however, improvements in terms of optical properties and fluidic connections, which led to the design of the 2nd generation culture system having an array of culture chambers and ‘plug-and-play’ fluidic connections. With optimized structural features and operational conditions, this system could be used to successfully culture tissue slices. The third generation culture chip was developed by adapting the 2nd generation culture chip to a versatile micro-

pump platform. However, culturing could not be performed properly due to unsuitability of the existing platform for tissue culturing resulting in problems when closing the chambers and removing the retained air. A slight modification is needed to resolve the problem for successful culturing. Finally, the 2nd generation culture system was tested for monitoring stem cell integration in brain slice culture under perfusion. Although the number of cells on tissue was decreasing during perfusion and further optimization is needed, the performed experiment and the obtained result pave the way for future experiments.

8.8. Outlook

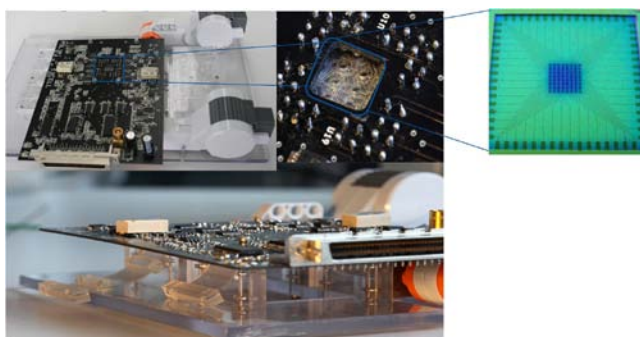


Figure 8.15: A cell/tissue culture system having an integrated electrode Microchip and potentiostat for measurements (e.g. potential recordings).

To go beyond optimization of individual parts of the general protocol for microfluidic tissue culturing and integration of stem cells as outlined by the experimental results obtained in research leading to the second part of this thesis, an automated system with integrated temperature and gas control is being fabricated. The design is a modified version of the platform used for the 3rd generation culture system in this work. After all the needed optimizations

of the new platform, the microfluidic culture chip will be integrated with an electrode microchip having 3D electrodes and a miniaturized potentiostat to conduct electrical measurements, e.g., potential recordings from tissue upon selective stimulation of integrated neuronal stem cells. Figure 8.15 shows an assembled prototype of the system with 3D electrode microchip in the inset.

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9. Overall Conclusion

Microfluidics can revolutionize research in the field of Medical diagnostics and biomedical sciences. The Lab-on-a-Chip concept is already being implemented in some companies. It will take the conventional systems to a brand new level.

9.1. Part I Chromosome analysis

Our main focus was developing a microfluidic system for metaphase FISH for chromosome analyses. In the process, two issues were mainly addressed: cost effectiveness and automation. Substrate suitability was examined for Metaphase FISH analysis. PDMS and COC substrates were suitable based on their auto fluorescent effect. COC has milky effect, which was a hindrance to observe the signals. PDMS is non auto fluorescent, but its surface property and gas permeability could be a problem for a PDMS based chip.

A closed chip is not good for chromosome spreading; rapid evaporation is a key factor in obtaining good chromosome spreads. Temperature treatment also has an effect.

The miniaturization of a metaphase FISH chip was shown in chapter 3. Based on our findings of chromosome spreading in a closed chamber, we designed a splashing device to obtain good chromosome spreads on a glass slide, which was then implemented to suit the micro FISH device. A successful metaphase analysis was performed on this chip.

Towards automation a polymeric chip was presented for obtaining chromosome spreads and metaphase analysis on a single chip. The chemical reagents for FISH analysis were stored in a PEI cartridge, which enabled the semi automation of the process.

But optimization of this technique is required for real-time applications. Passing all the optimization procedures, there is no doubt, that this platform will be very useful in routine diagnostics in clinical cytogenetic laboratories.

9.2. Part II Brain slice culturing

To the best of our knowledge, we have demonstrated for the first time a brain slice culturing in a perfusion based microfluidic system for more than two weeks. Tissue cultivation could be performed in a microfluidic chambers observable under microscope for time-lapse monitoring. The desired temperature in the culture system was maintained by a Peltier heater.

One of the major problems in tissue culture systems, occlusion of air when closing the culture chambers after tissue loading, has been solved with funnel shaped microstructure, effectively facilitating the removal of the air.

An attempt has been made to monitor grafting of neuronal stem cells into brain slices in a microfluidic tissue culture system. However, using the preliminary results as a starting point, new experiments have to be performed to optimize the procedures.

9.3. Outlook

Part I Chromosome analysis

Material suitability needs to be studied further based on auto-fluorescence, temperature and chemical resistances, surface properties, bio compatibility etc. This will be helpful for FISH, DNA, and protein analyses, as well as in immunological techniques etc. The automated Lab-on-a-chip concept is cost-effective and efficient to be implemented in medical diagnostics.

Single polymeric microchip for chromosome analysis was developed by investigating time and temperature variants on chromosome spreading. Complete automation still needs to be explored. The FISH signals can be easily read out by a microscope and corresponding software application.

Another important issue, reduction in assay time has to be studied. Hybridization process is the longest and time consuming step which could be fastened by the electro kinetic method. Applying voltage to the inlet and outlet and switching on the current from each side with regular intervals, will help the probe to move at a fast pace from the top to the bottom of chip surface where it hybridizes with the fixed chromosomes.

Accomplishing all these aspects, the microfluidic system needs be tested with a clinical sample for efficiency of this chip which could further be adopted in a clinical setup for pre- and post- natal diagnosis.

Part II Brain Slice Culturing

Technical aspects

The necessary technical modifications multichannel platform with micropumps will be implemented to facilitate a successful integration of tissue culture chips. The pump platform will be equipped also with a system for temperature and gas control to allow culturing of tissue slices on a microscope stage for continuous monitoring (modified 3rd generation system).

The significance of porous membrane as the culture surface in microfluidic tissue culture systems will be evaluated using the same kind of membrane material as in the inserts used for the traditional interface method. This test is expected show whether nutrient supply from underneath is necessary for extending the culture period over 3 weeks.

Flow rate during stem cell integration will be optimized using the modified 3rd generation system. Although computer simulations can be helpful to evaluate the sheer stress caused by different flow rates in culture systems with

different geometry, a successful protocol for stem cell integration has to be validated in a series of optimization experiments.

Applications

The microfluidic tissue culture system needs to be integrated with 3D electrodes for potential recordings from tissue upon selective stimulation of integrated neuronal stem cells. A new approach in neuroscience for selective stimulation of individual cells is optogenetics. This approach take advantage of cloning light sensitive ion channels from bacteria into, for instance, neuronal stem cells[160]. By exposing such genetically modified cells to light of certain wavelength, the cloned ion channels facilitate entrance of potassium ions against concentration gradients to polarize the cells triggering exocytosis. Hence, only the genetically modified cells are selectively stimulated and responses can be measured from the tissue. This approach can be implemented using a microfluidic system having both integrated 3D electrodes for measurements and inbuilt optical fibres for light induced cell stimulation.

List of publications

Peer Review Journal Publications

1. Vedarethinam I, Kwasny D, Dimaki M, Tumer Z, Silahtaroglu A, and Svendsen WE. Factors Affecting Chromosome Spreads on Polymeric Microfluidic Chip, *Manuscript prepared, yet to be submitted*
2. Kwasny D, **Vedarethinam I**, Shah PJ, Dimaki M, Silahtaroglu A, Tumer Z and Svendsen WE. Recent developments of microtechnology based methods for detection of chromosomal abnormalities *Manuscript prepared, to be submitted*
3. **Vedarethinam I**, Shah P, Dimaki M, Tumer Z, Tommerup N, and Svendsen WE. Metaphase FISH on a Chip: Miniaturized Microfluidic Device for Fluorescence in situ Hybridization. *Sensors* 10: 9831-9846, 2010.

Not Included in the Thesis

4. L. Sasso, **I. Vedarethinam**, J. Emnéus, W.E. Svendsen, and J. Castillo-León. "Self-Assembled Diphenylalanine Nanowires for Cellular Studies and Sensor Applications". *Journal of Nanoscience and Nanotechnology*, In press
5. Svendsen WE, Castillo-León J, Lange JM, Sasso L, Olsen MH, Abaddi M, Andresen L, Levinsen S, Shah P, **Vedarethinam I**, and Dimaki M. Micro and nano-platforms for biological cell analysis. *Sensors and Actuators A: Physical*

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6. Shah P, **Vedarethinam I**, Kwasny D, Andresen L, Dimaki M, Skov S, and Svendsen WE. Microfluidic bioreactors for culture of non-adherent cells. *Sensors and Actuators B-Chemical* 156: 1002-1008, 2011.
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Peer Reviewed Conference Publications

1. Vedarethinam Indumathi., Avaliani Natalia., Tønnesen Jan., Hansen J., Sabourin David., Dimaki Maria., Kokaia Merab., Dufva Martin., Svendsen W.E., Emnéus Jenny., Heiskanen Arto., Long-term brain slice culturing in a microfluidic platform. 15th International Conference on Miniaturized Systems for Chemistry and Life Sciences (**μTAS 2011**), Seattle, Washington, USA, Eds. J.P. Landers, A. Herr, D. Juncker, N. Pamme, J. Bienvenue, Vol. 1, 1560-1563 (**2011**). Type Poster presentation
2. Vedarethinam, Indumathi; Shah,Pranjul; Dimaki, Maria; Silahatoglu,Asli; Tumer,Zeynep Tommerup, Niels; ,and Svendsen,

- Winnie E.FISH on Chip: Chromosome Analysis on a Microfluidic Platform. 8th European Cytogenetics Conference, 2011, Porto, Portugal. Type: poster presentation
3. Vedarethinam, Indumathi; Avaliani Natalia; Tania; Tønnesen, Sabourin, David; Dimaki, Maria; Kokaia, Merab; Dufva, Martin; Svendsen, Winnie Edith, Emnéus, Jenny; and Heiskanen, Arto. Development of a Microfluidic system to Monitor Integration of Stem Cells in a Organotypic Brain Slice culture, 7th International Conference on Biomedical Applications of Nanotechnology, 2010, Berlin, Germany. Type: Poster presentation
 4. Vedarethinam, Indumathi ; Lange, Jacob Moresco ; Dimaki, Maria ; Shah, Pranjul Jaykumar ; Clausen, Casper Hyttel ; Svendsen, Winnie Edith ; Tümer, Zeynep ; Tommerup, Niels. FISH (Fluorescent in Situ Hybridisation) on Chip, The 12th Annual European Conference on Micro & Nanoscale Technologies for the Biosciences , 2008, Switzerland. Type : Poster presentation
 5. Vedarethinam, Indumathi ; Lange, Jacob Moresco ; Dimaki, Maria ; Shah, Pranjul Jaykumar ; Clausen, Casper Hyttel ; Svendsen, Winnie Edith ; Tümer, Zeynep ; Tommerup, Niels, FISH on Polymer, NanoBioEurope, 2008, Barcelona, Spain. Type : Poster presentation.

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